Origins of heterogeneity in Hypholoma fasciculare

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'After sternly calculating these possibilities (admitting that life was short, art long, opportunity instantaneous and experiment uncertain), he told himself that it was unworthy of a gentleman to be daunted by such petty calculations...'

Umberto Eco, 1995. ***The Island of the Day Before.***

'People always think something's *all* true. I don't give a damn, except that I get bored sometimes when people tell me to act my age.'

J. D. Salinger, 1951. ***The Catcher in the Rye.***

Abstract

The basidiomycete *Hypholoma fasciculare* (Sulfur-tuft fungus) was used as a model organism to study the origins and effects of genetic, developmental, biochemical and environmental heterogeneity on and in mycelial systems. Subcultures from *H.fasciculare* fruit-bodies gave rise to cultures with a wide range of mycelial organisations and phenotypes including normal dikaryon cultures, 'flat' dikaryon cultures with limited aerial mycelium, and homokaryotic cultures. Somatic genetic recombination was found to have occurred within these cultures when the mating-type genes were used as a genetic marker system. Biochemical analysis of the *H.fasciculare* cultures by HPLC showed a strong correlation between the production of hydrophobic metabolites and mycelial phenotype, with normal dikaryon cultures expressing the hydrophobic, mycelial-associated metabolites lacked by 'flat' dikaryons and homokaryon strains. Combative ability of *H.fasciculare* strains against other fungi was also associated with these metabolites. All cultures expressed a large hydrophilic metabolite peak that was found to be exported into the growth medium. A heterogeneous culture-system (the matrix-plate) was combined with image-analysis to analyse the growth and differentiation dynamics of *H.fasciculare* dikaryons in response to environmental heterogeneity. A 'flat' dikaryon was found to have reduced mycelial cord-forming ability and irregular growth kinetics when compared to a normal dikaryon. Translocation processes were observed occurring within *H.fasciculare* mycelia grown on matrix plates, these was quantified and correlated to a simple mathematical model. Metabolite production was found to be generally increased in high-nutrient compartments in matrix-plates. All of these results were discussed within the context of a theoretical model for mycelial differentiation that emphasises the

importance of oxidative stress and hyphal boundary chemistry in defining the mycelial phenotype.

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Chapter One

General Introduction

Section 1: Heterogeneity in living systems

Life fundamentally depends on energy flow, and nothing can flow without gradients. A gradient requires that two areas be different; so it is arguable that life depends absolutely on differences, or heterogeneities, in the world around it. In more specific terms, heterogeneity encompasses the differences and variations that underlie, challenge and develop from the living world.

The manifestations of biological heterogeneity can be chased up and down the ladder of scale from molecular to planetary. Scale, both in terms of time and size, is of paramount importance when considering these issues. The reference scale chosen for most of the work presented in this thesis is that of the individual organism, with forays 'upscale' into populations and 'downscale' into developmental patterns and genetics. To provide some context for this, the next section summarises some of the important types and scales of heterogeneity.

Environmental heterogeneity

The basic sources of biological heterogeneity lie in the physical structure that defines the biosphere. Free energy in the form of sunlight is not evenly distributed; it varies according to time of day, season, latitude and landscape. Water distribution depends on weather and geography. These are often thought of as large-scale processes, but clouds, weather and landscape all follow a fractal geometry (Gleick, 1987). This means that within many arbitrarily small regions there will be differences in light, moisture and nutrient concentration, and these differences may well be strong enough to affect living things. On top of the physical structure of the biosphere there is a biological structure. All organisms live and die within a context made of other organisms, a context of

predator, prey, invader, parasite or symbiont. Even organisms that do not interact in these terms are part of each other's physical surroundings. In the course of their existence, living things tend to concentrate useful resources within their boundaries and export waste products, so even initially homogeneous habitats become subdivided following colonisation.

Every organism has a set of physical and biotic circumstances within which it does the job of surviving and reproducing. The heterogeneity experienced at the 'organism's-eye' level will depend on the structure and scale of that organism. The organism's structure defines what *type* of heterogeneity is immediately important. The organism's scale (in space and time) defines which *levels* of environmental heterogeneity are immediately important. As an example, single-cell algae and trees are both structured to use light for energy, sharing *type* but not *scale,* whereas cheetahs and gazelles are (relatively) similar in size but eat different things, sharing *scale* but not *type*. From the organisms-eye-view, then, the world is divided into areas and times that it can thrive in and areas and times that it can't. As survival and reproduction depend on exploitation of the former and endurance or avoidance of the latter, heterogeneity in the natural environment is a fact that all living things have to deal with.

Basic organisational forms of life

Fundamental distinctions can be made between living things based on the concepts of unitary or modular structure and determinate or indeterminate growth. The distinction between modular and unitary organisms is based on their developmental pattern. Modular organisms develop by the repetition and elaboration of simple structural units, whereas unitary organisms have a single, functionally integrated body-plan. Mycelial

fungi and plants are regarded as modular organisms, whereas all vertebrates and insects, and most bacteria and yeasts, are unitary. Determinate growth occurs when organisms reach a certain mature size, then cease growth as part of their developmental program. This pattern is typical of mammals, insects, yeasts and non-filamentous bacteria. Indeterminate growth describes an open-ended increase in size and conservation of developmental potential throughout the lifespan of an organism. Examples of indeterminate growth can be seen in many plants, mycelial fungi and actinomycetes. It may seem from these categories that all modular organisms are by definition indeterminate, but this is not necessarily the case. Filamentous fungi can grow in self-limiting colonies and some plants grow to a developmentally determined endpoint (Andrews, 1992).

Species and population-level heterogeneity

The species is the most well-worn unit of biological heterogeneity. Species are traditionally defined as a pool of many similar, interbreeding individuals. The degrees of difference between species are usually attributed to divergence from common ancestors over evolutionary time under various selection pressures.

As is often the case, simplification can lead to understanding at one level and neglect at others. Species are composed of similar, interbreeding, individuals, but the similarities may be heterogeneously distributed and the interbreeding patterns variable. Populations, metapopulations and ecotypes are all terms used for sub-divisions within species that may represent levels of heterogeneity just as important to long-term evolutionary processes as the species. The 'tree of life' may well have a fractal structure, and 'species' may well be a unit meaningful only in terms of human choice

and time-scale. Particular scales of viewing the evolutionary process may bring out particular features, but evolution as a whole works on all different scales simultaneously.

Organism-level heterogeneity

Below the species, the next most significant level of organisation is the individual. As with many biological definitions, that of 'individual' seems to follow a sliding scale from the 'hard' end of mammals with well-defined body-plans and generations to the 'soft end' of ant colonies, clonal plants and fungal mycelia. Since this thesis will soon begin to enter the murky areas of mycology, the concept of the 'genetic individual' will be used here; a definition based on the body-plan derived from a single genome, however this body-plan is generated or distributed. Even with identical genomes, organisms will grow and develop in differing ways based on local environment and history. The variable challenges meted out by the environment have been discussed above, and these will always have an impact on the levels of heterogeneity within a population of individuals, whether genetically different or not. The genetically-derived component of this heterogeneity is the classic feedstock for Darwinian natural selection.

One important facet of heterogeneity between individuals of the same species has been described as 'alternative adaptation' (West-Eberhard, 1986). This refers to the ability of many organisms to generate very different phenotypes from the same basic genetic information in response to a 'genetic switch'. A classic example is the sex-determination system of crocodiles, in which sex is determined by the temperature at which the egg is incubated. Further whole-organism examples include the

tadpole/frog dimorphism and the differentiation of castes of social insects from the same genetic stock. Alternative adaptation may be seen as an extreme expression of epigenetic flexibility (see below). This last example (social insects) brings another factor into the consideration of individual-level heterogeneity, that of co-operation and integration between individuals. Such activities may act in concert with epigenetic specialisation to alter the pattern and range of variation within a population. This kind of social organisation can be seen in both the colonies of ants and fungi, if hyphae and ants are seen as individuals within the colony population. (Rayner and Franks, 1987).

Genomic and genetic heterogeneity within the organism

The genome refers to the sum of the genetic information (usually taken to mean DNA) within each cell of an organism. In eukaryotes this is partitioned into nuclear and organelle genomes and undoubtedly represents the densest concentration of information within the cell. Over recent years more and more levels of structure have been found within genetic regulatory systems. The basic unit of this structure is often taken as the gene. In the sense used here a gene is defined functionally as a single transcriptional unit, as opposed to the sense used by Dawkins (Dawkins, 1976) in which a gene is defined from the evolutionary standpoint of 'any locus with alleles that control a selectable feature.'

The coarsest patterns of genetic heterogeneity arise from the combinations of different genomes within the same cytoplasm. This has happened several times in evolutionary history as witnessed by the existence of mitochondria, chloroplasts and other organelles that show vestiges of a free-living past. This process may be in progress (or possibly stalled) in the case of the nitrogen-fixing bacteria that live in the

roots of leguminous plants; these 'bacteroids' seem to live in a half-world between organelle and organism (Brock *et al* 1994). If such associations are to survive there must be a considerable degree of co-operation between the two genomes, and this may eventually lead to the total integration and gradual loss of identity of the minor partner. In some cases such intracellular symbionts may have originated as the prey or parasites of primitive cells.

Genetic heterogeneity of a different kind arises when two equivalent, but different, genomes are merged by sexual processes to form a new diploid (or in fungi, heterokaryotic) organism. One of the classic benefits of this kind of heterogeneity is that different genomes are able to complement each other's deficits, leading to a positive synergistic effect on the fitness of the organism. However, there may also be costs to this process if genes functionally interfere or conflict with each other. In an interbreeding population, conflict-causing alleles of genes may well be strongly selected against, leaving a population of varied but 'harmonious' alleles. This process may not be as effective if the species is divided into metapopulations, where each may arrive at a different balance of alleles. If such populations are suddenly brought into breeding contact, there may be a clash between the two allele sets that could lead to variable and poorly co-ordinated genomes in hybrid progeny. Many of the consequences of integrating disparate genomes may reflect disturbances in the epigenetic organisation within the cell, as discussed below.

Epigenetic heterogeneity

Epigenetic processes are those which modify the expression of information existing within a given genotype. Most epigenetic alterations are reset by meiosis and are

therefore confined to one organism, some may be heritable. In terms of heterogeneity, epigenetics means that genomes identical at the DNA-sequence level can exist in very many different informational states determined by DNA methylation, chromatin structure and DNA-bound protein populations (Cedar and Razin, 1990, Jablonka and Lamb, 1995).

The degree of informational crosstalk that has been discovered between genes has given a new perspective to genetics; genes are now seen less as 'blueprints' for structure but increasingly as neurone-like elements in regulatory networks that can process information using inhibitory, excitatory and back-feeding connections. (Hunter, 1987, Pardee, 1994, Strohman 1994). The 'hard-wired' patterns of gene sequence define a pattern of channels through which epigenetic patterns can flow. Examples of this include the generation of the circadian rhythm in *Drosophila melanogaster* and *Neurospora crassa* by the genes *per* and *frq* respectively; this is due to the operation of a negative feedback loop in the transcriptional activity of the *per* and *frq* loci (Page, 1994). It has also been shown that the disturbance of epigenetic systems by the insertion of exogenous copies of nuclear genes can lead to dramatic 'metastable' gene activity patterns (Jorgenson, 1995). Epigenetics leads directly to phenotypic plasticity and responsiveness to the environment and it is absolutely required for differentiated, multicellular body-plans.

The evolutionary importance of epigenetic effects is closely tied to the pattern of germ-line differentiation. In organisms with an early segregation of germ-line and somatic tissue such as mammals, heritable epigenetic effects may be limited in scope. However, in organisms with a late segregation of germ-line and somatic tissue, such as plants and fungi, there is ample opportunity for epigenetic modifications to be acquired

and potentially passed on between generations. In indeterminate organisms such modifications may also be perpetuated 'horizontally' by the spread of ramets of the same genetic individual. It has been hypothesised that feedback between epigenetic and genetic information may have important consequences for such organisms over evolutionary time leading to non-random, possibly even gene-targeted genetic variation (Jablonka and Lamb, 1995). Such 'neo-Lamarckian' theories are intended to complement rather than undermine the established neo-Darwinian view, and have certainly increased the stakes in the attempt to understand epigenetic systems and their relationship with the genotype and environment.

Organism strategies for dealing with heterogeneity

The imposition of a heterogeneous environment on an organism will have important physiological and evolutionary consequences. The two most obvious strategies for coping with a changeable environment might be termed buffering and avoidance/exploitation. Buffering reflects processes by which physiology can adapt to cope with or exploit new circumstances, while effectively maintaining 'business as usual'; in other words, organisms make the best of what they've got. The complex homeostatic systems employed by most animals are an example of this; factors such as temperature, pH and osmotic potential are precisely controlled within the body despite exposure to wide environmental fluctuations. Buffering may extend out into the environment as the organisms uses a zone of control to ensure its security; micro-organisms may do this via antibiotic or enzyme release or the creation of mucosal or sclerotized boundaries made from polysaccharides, phenolics or other compounds. Larger organisms may build tunnels, nests or even air-conditioned office-blocks.

Avoidance/exploitation strategies can be used through space or time, and in harsh conditions may be seen as another way of dealing with changes outwith the tolerance of buffering processes. The 'static' avoidance strategy of dormancy involves avoidance across time, and is often employed when local circumstances are so unfavourable that the organism cannot function normally and must enter an resource-conserving or resistant state during the bad times; re-emerging when conditions improve. Spatial avoidance/exploitation strategies mean that organisms can physically move or grow away from unsuitable circumstances and towards favourable ones. This can be seen in even the simplest mobile, unitary organisms such as the flagellate bacterium *E.coli*, where random 'tumbling' is alternated with efficient straight-line propulsion (Brock *et al* 1994). Tumbling is engaged more frequently when chemoreceptors indicate the presence of nutrient molecules, with the result that the bacterial cells spend relatively longer in nutrient-high zones than nutrient-poor ones. If this behaviour is visualised as a trajectory, it can be seen that a relatively greater length of the trajectory fills space within high-nutrient areas as opposed to low-nutrient areas. This space-filling ability can be measured by various means and is can be quantified by 'D', the fractal dimension. The same considerations can be applied to the growth of modular organisms such as fungi or plants; the difference in this case is that the structure of the organism represents the 'solidified trajectory' of each of the growing points (meristems or hyphal tips) through the environment. Experiments to measure the variable space-filling capabilities of plants and fungi grown in heterogeneous environments have been carried out and in many cases confirm the simple prediction that space-filling ability increases in high-nutrient areas and decreases in low-nutrient areas (Wijesinghe and Hutchings 1996, Ritz and Crawford 1990).

Variable space-filling properties may often correspond to a need to maximise or minimise the boundary area exposed to resource-providing or resource-draining circumstances. Boundary-minimisation is best achieved by aggregating smaller structures together and/or adopting circular or spherical shapes. This means that shifts towards larger scales of organisation in biological systems are often driven by external factors such as resource depletion or a challenge by hostile or competitive organisms. Many examples can be drawn from many biological scales: Resource limitation drives the aggregation of *Dictyostelium* amoebae into the multicellular 'slug' body form; the hyphae of many higher fungi consolidate into mycelial cords if challenged by another fungus; birds and grazing mammals flock together when migrating or threatened by predators; humans also tend to gather together in times of trouble. These shifts in organisation have the important properties of minimising exposure to energy loss or attack and increasing the co-ordinative ability of the organism in question, enabling massed resources to be delivered to areas of threat or opportunity. In evolutionary terms such changes in the scale of biological organisation can open up enormous ranges of new diversity, as witnessed by the unicellular/multicellular transition of life on Earth.

Heterogeneity in relation to r and K selection

The terms r and K derive from the logistic equation of population growth, where r represents the reproductive power of the organism and K represents the carrying capacity of the environment. These terms have been widely used in a theoretical description of different ecological strategies, although supporting evidence for such a broad theory is patchy (Andrews, 1992). The theory can be summarised as follows:

When a fresh environment is colonised there is an initial phase of r-selection as organisms which reproduce rapidly dominate the resource base. As population densities increase towards the carrying capacity (K) of the environment, selection starts to favour organisms with greater qualities of resilience. As a crude simplification, r-selected organisms have a rapid, prolific life-cycle and small body-size, whilst 'K'-selected organisms have a slower life-cycle, large body-size and stress-tolerant or combative tendencies. K-selection is thought to occur in situations where there is a slow, continual supply of nutrients within a relatively stable environment. This would typically occur in diverse, established ecosystems or in high-stress environments of extreme temperature, toxicity *etc*. In broad terms, K-selected organisms must deal with heterogeneity in terms of competing organisms and environmental stress.

The circumstances that favour r-selection are the transient availability of large nutrient supplies, often through the processes of disturbance within an established ecosystem. Examples of such circumstances include clearings caused by a tree-fall or fire in a forest; the dropping of dung by large animals and the carcass left after the death of an organism. Many parasitic organisms are considered to be r-selected because they have only transient access to hosts, which then supply a rich source of nutrients for proliferation. The heterogeneities faced by r-selected organisms are therefore chiefly those of resource distribution.

Model organisms : Weeds and vermin?

The organisms that have been most used for biological research, particularly genetic research, have tended to be species with small, easily maintained individuals and rapid, prolific life-cycles. The exceptions to this rule are often domesticated organisms such

as cereals and livestock with highly selected genomes. Plant genetics has concentrated on *Arabidopsis*, animal genetics has concentrated on fruit-flies and mice and fungal genetics has concentrated on yeast, Ascomycetes and the more ruderal of the Basidiomycetes. It could therefore be argued that the qualities most sought-after in model organisms have therefore restricted much research to the weeds and vermin of the environment, or to be more precise, r-selected organisms.

The concentration on r-selected organisms has been inevitable, and given the resources available for research, made perfect sense. However, problems may arise when generalisations are extended from these model organisms and applied uncritically to the wider living world. Many parallels will be valid, but some will not, and if K-selected organisms are treated the same way as r-selected organisms in the laboratory then a misleading picture may be developed of their nature and capabilities.

Section 2: Heterogeneity in mycelial fungi

Species and population-level heterogeneity

All mycelial fungi are heterotrophs; that is, they live by absorbing organic compounds ultimately derived from other organisms. Within this broad bracket there are distinctions in feeding mode commonly referred to as saprotrophic, necrotrophic and biotrophic; saprotrophs feed on dead or decaying organic matter, necrotrophs acquire the same material by actively killing a host, and biotrophs 'skim' live hosts for resources. It is possible for fungal species to blend or alternate between these options; some fungi show an alternation or combination of feeding modes. (Cooke and Rayner, 1984). The heterotrophic lifestyle is sufficiently flexible for the fungi to have pervaded practically every environment capable of supporting life on the planet at scales ranging from the microscopic to the geographic.

Heterogeneity within fungal species

In a heterogeneous environment, different allopatric or sympatric populations within the same fungal species often have different environmental conditions to contend with. This may lead to the development of different physiological and genetic adaptations within these populations, leading to a 'metapopulation' structure within the species. Phenotypic variations between the allopatric populations (Schmidt and Liese, 1978) and sympatric populations (Wildman, 1995) of fungi have demonstrated important intra-species physiological and biochemical differences. The underlying (epi)genetic differences responsible for much of this variation can be demonstrated if fungal individuals from allopatric populations of the same species are mated together. Such

mating reactions often result in unstable and unpredictable mycelial phenotypes and the release of large amounts of unusual secondary metabolites (Ainsworth and Rayner, 1989, Ainsworth *et al* 1992); responses that may be interpreted in terms of genomic conflict between functionally semi-compatible genomes. These results indicate that considerable (epi)genetic heterogeneity can develop if significant 'horizontal' boundaries are present that divide a species into populations.

Life-cycle stages

Different fungal life-stages can have very different phenotypes. The genome of a basidiomycete at any one time is typically made up of one or two nuclear types and one mitochondrial type. Variations occur at this level between homokaryons with one nuclear type (plus one mitochondrial) and heterokaryons with two nuclear types (plus one mitochondrial). This is associated with morphological switches in many fungi, such as the yeast/ mycelial switch in *Ustilago maydis* and the fast-effuse/slow-dense mycelial switch in *Hypholoma fasciculare*. Such switches can be considered as alternative phenotypes and have the important consequence that different life-cycle stages of fungi may inhabit different ecological niches and be subject to different selection pressures. For example; the phenotype most suitable for an established heterokaryotic mycelium is not necessarily the best phenotype for a newly-germinated homokaryotic spore. This may mean that different life-cycle stages will have different strategies within the r/K selection spectrum (Andrews, 1992).

Variation between fungal individuals

In terms of genetic variation, the degree of difference between the genotypes of individuals within a local population will depend upon the frequency of genetic recombination and the outbreeding/inbreeding strategy employed (Rayner, 1990). The presence of different fungal genotypes is often signalled by vegetative incompatibility, where different mycelia recognise and react to each other by forming degenerate, pigmented interaction zones. Such territoriality can lead to the compartmentalisation of resource units by warring mycelia and lead to the superficially paradoxical result that heavier inoculations of spores will lead to reduced assimilation of resource-units as boundary zones proliferate and individual fungal territories diminish (Coates and Rayner 1985*a*).

In addition to (epi)genetic variations, differences between individuals also relate to acquired physiological factors such as age, size and historical exposure to the whims of the environment. The population of every species includes individuals of various ages including the young, mature and old. In an indeterminate organism age may often be linked to body size, the measure of which may be complicated by the distribution of a genetic individual in the form of ramets. Ageing is generally linked to progressive genetic damage and dysfunction. This may not be as important for totipotent, indeterminate fungal mycelia where each part of the individual can act as a focus for new growth; this is evident in the large size and age achieved by some mycelia. Fungi can survive and grow for hundreds of years as lichens and to take up hectares of space and tonnes of biomass as saprotrophic networks on forest floors (Smith *et al*, 1992). Despite this, indeterminacy represents a potential rather than a constant, and senescence processes are often capable of curtailing the growth of

mycelial fungi (Marbach and Stahl, 1994). This may be particularly true of resource-unit restricted fungi which, although potentially indeterminate, will seldom exceed a certain size or age defined by the resource-unit quality and dimension. Such fungi may not require long-term mycelial persistence and may therefore acquire characteristics that undermine it without evolutionary cost. This may eventually lead to the conversion of the fungus from an indeterminate to a determinate development pattern.

Differentiation within the fungal individual

The higher mycelial fungi have been described as living on the edge of the individual/population definition (Cooke and Rayner, 1984). The mycelium can act as a concerted whole, generating higher-order structures and translocating nutrients, but it is also a population of potentially totipotent compartments. The balance between these levels of individuality is one of the most fascinating aspects of the higher fungi, and colonial organisms in general.

Organisational shifts in scale and pattern can often be seen within fungal mycelia. The greatest exhibition of differentiation by the higher fungi, and the one that usually accounts for their visibility in the environment, is the formation of fruit-bodies. These structures are a determinate phase of the fungus, produced for the purposes of sexual reproduction and the dissemination of spores. Fruiting is generally triggered by nutrient limitation in combination with other factors such as light exposure and temperature changes. The exact circumstances vary from species to species and it can be appreciated that the control of such an important function will be under niche-specific selection pressures. Fruit-bodies are highly differentiated structures and have

attracted research into the genetic control of their initiation development (Dyer *et al*, 1992, Wessels 1992).

Mycelial cords are connective structures produced by some higher fungi, varying in form between loose hyphal aggregations and highly differentiated, melanised 'roots'. Cords fulfil several functions, including efficiently foraging for new resources, crossing regions unfavourable for growth, invading other fungal territories and translocating resources to developing areas of mycelium (Boddy, 1993). Mycelial cord formation can be triggered by unfavourable circumstances including antagonistic organisms and mycelial interaction with hydrophobic substrates, or may occur within mature mycelia when the local nutrient supply is depleted. As sessile organisms, mycelial fungi must deal with such resource depleted zones either by forming dormant, resistive structures to wait out the bad times, or escaping by sporulation or mycelial (cord) growth. These last two strategies differ in the scale and pattern of resource allocation. A spore may be compared to a 'fungal bullet', endowed with a limited amount of energy and a single chance to find its target of a suitable environment. The foraging cords of the higher fungi are more like 'mycelial tanks', able to manoeuvre, persist in difficult circumstances and bring enough resources to bear to displace opponents from productive territory.

Fruit-body and cord formation both represent highly visible shifts in organisation, but there may be less obvious (but no less important) differentation processes occurring at smaller scales within the much-ignored 'cotton-wool' of the mycelium. Mycelia have been described as possessing an 'indeterminate embryology' (Rayner and Franks 1987) that is capable of adapting to local circumstances and creating efficient solutions to the problems of integrating assimilative, explorative and

reproductive functions. An understanding of this kind of mycelial organisation is vital if the role of fungi within ecosystems is to be properly understood, as has been emphasised in the context of interactions between mycorrhizal fungi and their plant partners (Cairney and Burke, in press). One of the most important qualities of a mycelium is its degree of interconnectedness. This is controlled by the number of anastomoses between hyphae and the resistance to flow along their length. Without anastomosis, fungi would be limited to a radial growth-form which would have little ability to redistribute resources. Such redistribution of nutrients has been shown to be an important factor in the development of mycelial morphology, with wide degrees of variation shown in the redistributional ability of different fungal species when grown in heterogeneous split-plate cultures (Olsson, 1995). Other differentiative processes within the mycelium may relate to the amount of insulative material in the cell wall, *i.e.* how well the cytoplasm is connected with its immediate environment and how deformable this boundary is. This seems to be particularly important in the differentiation of aerial as opposed to substrate hyphae (Wessels, 1993) and may act as a powerful way of controlling mycelial patterns (Rayner *et al* 1994).

Genomic heterogeneity

Genetic instability and somatic recombination processes have been documented in many cases in many fungi, both in nuclear and mitochondrial genomes. These range from extensive meiotic-like recombination down to the specific transfer of small regions of DNA (Frankel, 1979, Leonard and Dick, 1994). One of the main processes that can give recombinant genomes in fungi has been termed parasexuality, and occurs frequently enough in some species to be used as a genetic mapping tool. Parasexuality

involves the formation of a transient diploid nucleus which then undergoes a series of chromosome losses and reorganisations, finally generating an array of stable recombinant haploid nuclei (Caten, 1981). Levels of parasexual recombination can be increased by ionising and ultraviolet radiation, along with various chemical treatments. Somatic recombination processes can also occur when different, incompatible fungal individuals are brought together. This has been recorded in *Agaricus bisporus*, where recombinant genotypes were recovered from the interface zone between two different heterokaryons without the use of any special forcing or selection procedure (Xu *et al*, 1996).

In addition to parasexuality, many changes in chromosomal size and complement can occur in fungi. Studies of various fungi have shown that there is generally a high level of karyotypic stability under normal vegetative growth, but that changes in chromosome length and structure can occur during meiosis (Zolan, 1995). Some fungi have also been reported as possessing 'dispensable chromosomes' which may loosely be compared to plasmids in bacteria. Chromosomal length changes occurring during mitotic growth usually reflect alterations in the copy number of rDNA sequences and may represent an adaptive mechanism, as rRNA production can be a limiting factor in fungal growth. This, and more generalised karyotypic instability, is increased by growth under restrictive or non-standard nutrient regimes.

In addition to the nuclear genome, fungal mitochondria have been found to be prone to various genetic rearrangements, additions and deletions. Many fungi are host to mitochondrial plasmids which may or may not be homologous to nuclear or mitochondrial genomic DNA. Some of these elements are capable of causing genetic instability within the mitochondrion that can lead to a loss of function and the

degeneration and senescence of the host fungal strain (Marbach and Stahl 1994). Many elements are mobile between different fungal populations and possibly even species, and may be thought of as genomic parasites (Arganoza *et al* 1994). The effects of such an entity within a fungal genome may be far-reaching; for example, there is evidence that such a mitochondrial element may be involved in mating-type switching in *Stereum hirsutum* (Watkins and Ramsdale unpublished data).

Epigenetics

Most epigenetic processes have been found in organisms with a relatively large number of differentiated cell types (e.g. mammals), and relatively few have been reported in the higher fungi, although it is arguable that this may be a consequence of the different outlooks and directions of effort in the two fields of research. DNA methylation in higher fungi has been found, but so far it has only been associated with repetitive DNA elements, rDNA genes and multiple-copy transformation (Li and Horgen 1993, Wilke and Wach, 1993), none of which seem to play an important part in development. Some evidence does, however, exist for epigenetic processes in fungi. Fungal nuclei passaged through different cytoplasmic backgrounds have been shown to acquire imprints that affect their future behaviour. (Rayner *et al*, 1995) and complex effects of chromatin state on gene expression (position-effect variegation) have been documented in yeast (Aparacio and Gottschling, 1994). These findings suggest that there may be scope for further work on the epigenetics of fungal genomes.

Secondary Metabolites

Filamentous fungi produce a huge diversity of secondary metabolites; by 1983 at least two thousand different chemical structures had been recorded and the number is steadily increasing (Bennet, 1994). Secondary metabolites have been used as taxonomic markers between fungal species (Whalley, 1995) and variations in metabolite profiles have also been found between different populations of the same species (Wildman, 1994). Secondary metabolites have often been defined as those which are non-essential to the normal growth of the organism and tend to be produced when growth stops. This kind of definition is questionable on several counts when considering K-selected filamentous fungi. One of the key questions to ask is what is meant by 'normal growth' in the first place; in the context of laboratory experimentation it would seem to mean exponential, unrestricted growth in a nutrient-rich, axenic environment. This is a highly artificial homogeneous situation that may bear little similarity to the heterogeneous ecological context within which most fungi, especially K-selected fungi, operate. At best, these circumstances may occur briefly following disturbances in a local environment and are swiftly exploited by r-selected organisms, which may only experience a nutrient-limited and combative environment briefly before dispersal mechanisms take over. In the attempt to understand the functions of secondary metabolism more fully it may be useful to redefine 'growth' as 'growth in the environment' rather than 'growth in the laboratory'.

Section 3 : An integrated approach for the study of morphological heterogeneity in fungi

Recently, attempts have been made to derive a wide-ranging model framework for the study of mycelial growth and differentiation (Rayner *et* al 1995, Rayner 1996, Watkins *et al* in preparation). These hypotheses are based on simple thermodynamic principles of energy loss and gain coupled with the processes of oxidation-state control and secondary metabolism within fungal mycelia. Predictions made from this model framework agree with many of the observations made of the behaviour of mycelial fungi and are well-adapted to considering the origins of heterogeneity in mycelial fungi.

Oxidation state and free-radical generation

The oxidation state of a cell refers to the balance of reduced and oxidised compounds within it; this balance is a function of the respiratory activity, nutrient intake and gaseous environment of the cell. During the process of aerobic respiration, nutrients are taken in as relatively reduced carbon compounds which are then oxidised to provide energy and reducing power for cellular functions. This is achieved by the generation of proton gradients across mitochondrial membranes by the operation of the electron transport chain. High-energy electrons are made to do the work of pumping protons against the gradient as the electrons are passed down a chain of metal-ion containing carriers. The protons and electrons are eventually recombined with oxygen to form water.

The high-energy electrons do not always stay within the bounds of the respiration process, however, and various free-radical chemical species, including many reactive oxygen species (ROS), are the result. Free radicals are molecules with unpaired electrons; many are highly reactive, short-lived chemicals that are capable of causing significant damage to cellular components. The importance of free-radical damage and containment to the survival and proper functioning of the cell has become increasingly evident in recent years (Halliwell and Gutteridge, 1989). It has been seen as increasingly likely that many disparate kinds of cell-stress such as heat-shock, metal toxicity, starvation and desiccation are ultimately translated into oxidative stress involving increased free-radical activity (Davidson *et al* 1996, Meyer *et al* 1995). Mitochondrial dysfunction is a particularly strong sources of increased free-radical flux; such dysfunctions have similar effects in fungi whether occurring naturally due to genetic processes (Marbach and Stahl, 1994) or induced experimentally by the addition of metabolic inhibitors (Griffith *et al* 1994*a*)

Although frequently detrimental, free-radical production is also harnessed by some organisms for specialised functions. In the phagocytic white cells of the mammalian immune system free-radical production (that of superoxide anion radicals) is used to destroy phagocytosed infecting micro-organisms (Jones, 1994). Plants synthesise lignin, an important structural polymer, by randomly assembling various phenolic radical subunits. This effort is frequently reversed by the white-rot wood-decay fungi, which metabolise lignin by attacking it with radical-generating peroxidases and phenoloxidases.

Fungal differentiation as a response to oxidative stress

If oxidative stress is seen as a barometer for the general well-being of a cell, then it may be reasonable to expect that there will be cellular adaptations for reacting to and coping with such stress. Moreover, if oxidative stress is as fundamental to terrestrial fungi as has been claimed (Watkins *et al* in preparation), then it might also be expected that these adaptations will involve the most important and visible kinds of differentiation processes seen in mycelial organisations. The mechanisms available to a mycelium to counteract oxidative stress vary both in operational scale and according to circumstance, and are summarised below.

The simplest way for an aerobically-respiring organism to avoid oxidative stress is to maintain a high input of reduced carbon catabolites to the respiration chain, a strategy that has been termed 'gas-guzzling' (Rayner). This converts the hyphae into a sink for oxygen, which is consequently held at lower levels within the cytoplasm. This strategy will only succeed as long as there are plentiful supplies of reduced resources. The mycelial organisations suitable for this strategy would be high surface-area, frequently- branching, mycelia with gas-permeable hyphal boundaries. This organisation is typical of the substrate mycelium of r-selected fungi growing in a nutrient-rich site.

When the supply of reduced carbon is no longer sufficient to support 'gas-guzzling', oxidative stress levels will increase and various coping mechanisms will be brought into play. Some of these deal directly with free-radicals in the cytoplasm and include enzyme systems such as superoxide dismutase (SOD) and catalase (which remove ROS) and the production of intracellular free-radical scavenging metabolites such as ascorbate and glutathione. The SOD/catalase enzyme system has been shown

to have a crucial role in resisting heat-induced oxidative stress in the yeast *S.cerevisiae* (Davidson *et al*, 1996). Despite such mechanisms, a 'hyperoxidant state' may eventually arise within the mycelium. Further lines of oxidative stress defence may operate by reducing the influx of oxygen at source. The mechanisms proposed involve the addition of various kinds of gas-impermeable and oxygen-scavenging insulation in the form of crosslinked proteins, polypeptides or phenolics to the mycelial boundary; the hyphal wall and membrane. These modifications may result in the mycelial differentiation associated with the emergence of aerial hyphae; evidence for the correlation of the hyperoxidant state with aerial mycelium emergence has been found in *Neurospora crassa* (Hansberg and Aguirre, 1990).

It might be argued that diffusion of oxygen from the atmosphere would overwhelm many of the coping mechanisms proposed here. However, the mycelium of most terrestrial fungi grows within solid substrates of limited porosity where the gaseous regime may not necessarily be atmospheric-indeed, where oxygen availability may be a limiting factor. The boundary-sealing and sclerotisation processes occurring at the interfaces between the substrate and the atmosphere may help to partially seal out the potential flood of atmospheric oxygen and maintain a more limited oxygen regime. Evidence that oxygen levels can be markedly reduced by mycelial action comes from studies of *Phanerochaete chrysosporium*, a white-rot basidiomycete, where it was found that in undisturbed liquid cultures grown in a 100% oxygen atmosphere there was no detectable oxygen more than 2mm underneath the mycelium of the fungus. (Leisola *et al* 1983)

Soon after the emergence of aerial mycelium comes the point at which the dispersal mechanisms of r-selected fungi come into play, most commonly by

sporulation. The immediate resource base has been exploited and the invasion of more combative organisms is probably imminent or actively underway; so the 'r-selected' response is to shunt a high proportion of available resources into propagation; the success of this depending on the size of the mycelial territory acquired by primary resource capture. Beyond this point is the region where the superior differentiation abilities of the K-selected higher fungi come into their own in the fields of secondary resource capture, combat, defence and foraging.

Secondary metabolism and oxidative stress

Secondary metabolites are a diverse array of small, often cyclic or polycyclic, biochemicals produced by many fungi, plants and bacteria under conditions of nutrient scarcity, competition and defence. The definitions that have been attempted for 'secondary metabolism' are varied. Most centre around the concept of synthetic pathways that are not necessary for growth and are only activated after it has ceased (Bennet, 1994). Further elaborations of the definition include factors such as the production of biochemicals in closely-related, often unusual, structural 'families' limited to small taxonomic groups of organisms, and derivation from 'general' primary metabolites via unique pathways.

Many secondary metabolites have potent and unpredictable biological activities and their investigation has been largely driven by the practical (and profitable) uses that have been found for them. The balance of investigation into these compounds seems to have been skewed in the direction of '*what can be done with them?*' and '*what is their structure?*'. This is probably due to the outlook of the industry-driven screening programs which have uncovered the majority of novel metabolites. There seems to be

much less attention paid to the biological question of '*what are they there for?*'. Since secondary metabolites have such diverse and often ill-defined functions, this question has fallen into the provinces of speculation and has, seemingly, disappeared from the usual considerations of experimental mycology. The model for fungal differentiation in response to oxidative stress presented above opens up a new context for considering the overall function of secondary metabolites. Secondary metabolism is stimulated under conditions likely to cause oxidative stress and the secondary metabolites extracted from some higher fungi have been shown to possess remarkable free-radical scavenging abilities (Watkins unpublished). Such metabolites may be sequestered within the cytoplasm as a protective buffer, released into the immediate environment, or polymerised by phenoloxidase enzymes at the hyphal boundary to serve as insulants. The structural requirements for free-radical scavenging activity are not narrow, and so there is potential for the evolution of secondary, more specialised functions for any such compound.

Secondary metabolites may not be essential for assimilative growth *per se*, but they may be absolutely required for persistence and survival in the combative, high-stress environment experienced by more K-selected fungi. Consideration of this factor has lead to the productive targeting of metabolite screening programs in at least one case (Gloer, 1995). The prevalent concept of secondary metabolism being engaged when growth stops hides a large assumption; this is that all of an organism's structure is in the same state at the same time. This may rarely apply to an extended mycelial organisation; while one part of a mycelium ceases assimilative growth another part may be exploring or exploiting new resources. Viewed from this angle, secondary

metabolites in the more K-selected fungi may be seen as taking part in an ongoing process of mycelial differentiation, rather than as products of a 'terminal' state.

Oxidative stress and fungal enzyme systems

A class of fungal enzymes, the phenoloxidases (including laccases and catechol-oxidases), have been repeatedly implicated in processes relevant to fungal differentiation and morphogenesis. These metal-containing enzymes use molecular oxygen to oxidise a wide range of phenolic compounds, and the correlation of phenoloxidase activity with developmental events and externally applied stresses make these enzymes important to the oxidative-stress model. Some of the most straightforward cases of differentiation involving phenoloxidases involve the synthesis of pigment compounds in fruit-bodies and spores, where these enzymes are responsible for the final steps in the synthesis of melanin-like compounds (Law and Timberlake 1980, Vnenchak and Schwalb 1989). Melanin production is also initiated by some fungi in response to copper toxicity, with the melanin forming thick layers within the hyphal cell walls (Tonthat *et al* 1995); this is particularly interesting considering that laccases are copper-containing enzymes (Thurston, 1994). Patterns of phenoloxidase activity have been found to change within the mycelium as a whole during the initiation of fruiting in *Agaricus bisporus* and in response to light in *Schizophyllum commune* (Wood and Goodenough 1977, De Vries *et al* 1986). Such enzymes are also induced during combative interspecific reactions between *Hypholoma* fasciculare and other basidiomycete fungi. (Griffith *et al* 1994 a,b). Data on the location and control of these enzymes has added to the evidence of their importance in responses to oxidation state. Oxidation-state sensitive control elements have been found upstream of a laccase gene

in *Podospora anserina* (Fernandezlarrea and Stahl, 1996) and a laccase of *Coprinus congregatus* was found to be specifically located on the cell membrane (Kim and Choi, 1995). The picture that emerges from this mass of data is that phenoloxidase enzymes are often in the right place at the right time and are responsive to the right stimuli to fulfill a very important role in the boundary chemistry of fungi.

Most chemical processes are two-way streets, and free-radical chemistry is no exception to this rule. The metabolites and enzyme systems used to reduce free-radical damage to the hyphal contents and to build up insulative, polymerised barriers are capable of being turned around and used instead to degrade stable structures outside the hyphae as part of assimilative or pathogenic processes. One of the best examples of this is the degradation of lignin in wood by the white-rot fungi. Lignin is a random polymer formed by the random assembly of phenoxyl radicals, and its lack of repeating structure makes it a difficult target for conventional forms of enzymic degradation. White-rot fungi are capable of efficiently degrading lignin as part of the decay process, this is due to the activity of a complex of enzymes including manganese and lignin peroxidases and the phenoloxidase enzyme laccase (Rayner and Boddy 1988, Paterson and Lundquist, 1985). Substantial inputs of oxygen and hydrogen peroxide are required for ligninolysis; the latter may be supplied by oxidation of glucose released from degraded cellulose. The detailed biochemistry of ligninolysis is complex and involves autocatalytic processes, where free radicals generated by enzyme attacks on the lignin chain themselves assist in further breakdown of lignin structure. In terms of the oxidative stress model, one of the most interesting aspects of ligninolysis is the transcriptional control of the peroxidase and laccase enzymes. In at least one case these have been shown to be induced by heat-shock, metal ions and oxidative stress

(Brown *et al* 1993, Li *et al* 1995,). This indicates that the same stimuli that induce free-radical absorbing enzymes within the mycelium may induce the formation of free-radical generating enzymes outside the mycelium. Such distinctions emphasise the importance of context in the function of these enzymes.

Figure 1.1 : *Hypholoma fasciculare* fruit-bodies (approx. 2x life size)

Section 4 : The present study

Origins of heterogeneity in *Hypholoma fasciculare*

Introduction

Hypholoma fasciculare, known commonly as the 'sulphur-tuft mushroom', is a wood-decaying basidiomycete fungus frequently found in North European woodlands. Bright yellow *H.fasciculare* fruit-bodies (**Figure 1.1**) can be seen in clusters on decaying tree stumps. 'Frequently found' may be an understatement; one estimate has *H. fasciculare* listed as a find in half of all fungal forays, higher than any other species. (Phillips, 1994).

The genus *Hypholoma* is widespread, existing in both the Northern and Southern hemispheres (Miller and Pearce, 1996). Six species are described in European fungal identification books. Historically, a subdivision *Naematola* was created for the 'fleshy' *Hypholomas* (*H. fasciculare* and *H. sublateritium*), this lingers on in the chemical names of several *H.fasciculare* metabolites, the naematolins. *H.fasciculare* is readily cultured in the laboratory; a feature it shares with many other saprotrophic, as opposed to biotrophic, basidiomycetes.

Life-Cycle

In common with most basidiomycetes, *H.fasciculare* mycelium has two distinct mycelial forms, one termed homokaryotic and the other dikaryotic. These names refer to the types and organisation of nuclei within the mycelia, described in detail below. In summary, the germination of basidiospores gives rise to haploid homokaryotic mycelia. Homokaryons with compatible mating-types fuse and give rise to a dikaryotic mycelium. This is not a diploid phase, but contains the nuclei from both homokaryons in a close heterokaryotic association. When the dikaryotic mycelium forms fruiting bodies the two nuclear types finally undergo karyogamy and meiosis in specialised cells termed basidia. The meiotic products are liberated as haploid basidiospores, completing the cycle.

The homokaryotic phase of *H.fasciculare* is a mycelium containing a single nuclear type, usually derived from the germination of sexual basidiospores. One or more of these nuclei are partitioned into hyphal compartments defined by dolipore septae. Asexual spores (termed dry arthrospores or oidia) are often produced abundantly by the aerial mycelium, these are formed by the segmentation of specialised

aerial hyphae. In culture on 2% malt agar at 20°C, homokaryons extend radially by 1-2mm per day, usually with a right-angle hyphal branching pattern that produces a dense mycelial mat. The aerial mycelium developed by homokaryons can be very thick. Pigmentation is usually absent or limited in homokaryons; its presence is often associated with degenerate or senescent colonies. After the initial homokaryon colony form is established there is usually little subsequent change in the aerial mycelium or pigmentation of the colony.

Dikaryotic mycelium emerges after the fusion of two mating-type compatible homokaryotic mycelia and has a very different ultrastructure. It contains the two homokaryon-derived nuclei closely arranged in complementary pairs, each pair strictly partitioned into one hyphal compartment. As the nuclei divide, the maintenance of the nuclear pairing is associated with the formation of a clamp-connection. A short curving hypha termed a hook cell grows out from the hyphal wall next to the dividing nuclei and fuses, sub-terminally, to the main hypha. One of the daughter nuclei migrates through this 'bypass' and enables the re-organisation of the four daughter nuclei into two complementary pairs. These two pairs of nuclei are then recompartmentalised by the sealing of the hook cell and main hypha by dolipore septae. Clamp-formation has interesting organisational consequences. A hyphal compartment in a homokaryotic mycelium has one septal-pore connection with each of its neighbouring compartments, whereas a dikaryotic hyphal compartment has two, the main-hypha pore plus the clamp-connection pore. This parallel-resistance arrangement may explain the relatively increased translocational ability of dikaryons over homokaryons. *H.fasciculare* dikaryons cultured on 2% malt agar at 20°C grow faster than homokaryons, with a radial extension rate of 2-4mm per day. Dikaryotic hyphae typically branch at acute

angles and often aggregate into fans and bundles, giving the colony a fine, silky texture. This mycelium initially grows out white and relatively flat and then develops yellow to orange pigmentation upon ageing, often with the formation of aerial mycelium aggregates and cord structures. Very occasionally, small fruit bodies may form on cultures after about three months, but this is neither predictable or reliable under standard laboratory conditions. The aerial mycelium of dikaryons does not usually produce oidia, although a few can sometimes be recovered, possibly due to an occasional breakdown of dikaryosis in aerial hyphae.

The ecology of *H.fasciculare*

The dikaryon is the most persistent life cycle stage of *H.fasciculare*; in woodland soil its dikaryotic mycelium form a network of mycelial cords that forages for resources ranging from large pieces of wood down to leaf litter (Dowson *et al* 1988 I). Such cord systems extend from one resource base to another and may be very large; artificially inoculated *H.fasciculare* mycelia formed networks covering several metres after two years (Dowson *et al* 1988 II) and such networks may continue expanding for decades or longer.

In the ecological terms previously discussed, *H.fasciculare* is a combative, K-selected organism. Superficially, a wood-decaying fungus living on the forest floor may seem to be in a r-selected, disturbance-led ecology as pieces of woody substrate drop into its locality from the tree canopy. However, these resource units are highly likely to already support populations of fungi; saprotrophs, pathogens or endophytes of the tree that have already had a substantial time to establish themselves in the attached wood. (Cooke and Rayner, 1984). Combative ability is therefore important to a forest-floor

wood-decaying fungus if it is to gain access to these pre-colonised resource units. Two strategies have been discerned amongst such K-selected fungi; 'defenders' and 'aggressors' (Cooke and Rayner, 1984). The 'defender' strategy adopted by fungi such as *Daldinia concentrica* and *Xylaria hypoxylon* seems to involve primary resource capture by rapid hyphal growth followed by protection of those resources by structures such as crusts and pseudosclerotial plates (Rayner and Boddy, 1988). The 'aggressor' strategy relies on secondary resource capture; the invasion of wood-based mycelial territories held by other fungi. Fungi that use this strategy often have a slower mycelial extension rate but are able to mass considerable inoculum potential at a target site by using strong translocational abilities derived from mycelial cord systems. *Phanerochaete velutina, Phallus impudicus* and *H.fasciculare* are examples of fungi that use this strategy. In culture and field experiments *H.fasciculare* and other *Hypholoma* species have been shown to be adept in displacing and invading the mycelial territories of other fungi. (Pearce *et al* 1995, Holmer and Stenlid 1996, Griffith *et al* 1994*a*).

Mycelial cord formation in H.fasciculare

Hyphal aggregation and formation of cord structures are an important characteristic of *H. fasciculare* when considering its physiology and ecology. Both homokaryons and dikaryons of *H. fasciculare* show a tendency to form hyphal aggregates. However, this ability only seems to be fully expressed in the dikaryon, where cord formation may be induced by several factors. One of the strongest inducers of cord-formation is the presence of other fungal and bacterial competitor species. (Griffith *et al* 1994*a*). Cords induced in this manner are able to overrun and infiltrate fungal and bacterial colonies,

eventually leading to the death of the other organism. Although antagonistic reactions are the strongest inducer of cord-formation, cords are also produced in pure cultures of *H.fasciculare* upon ageing, probably in response to nutrient exhaustion and the requirement to recycle resources within the mycelium. Such cords are also often initiated at the points where the mycelium encounters the plastic walls of culture plates, or by small areas of damage to the mycelium. Cord formation may also be artificially induced by the addition of chemicals, particularly metabolic uncouplers such as DNP which short-circuit the proton gradient of the mitochondria (Griffith *et al*, 1994*a*). Cord formation in *H.fasciculare* may therefore be a response to the internal oxidative state of the mycelium, which could explain the many different stimuli that can initiate it.

Homokaryons of *H.fasciculare* are also able to generate patterns of hyphal aggregation, but seem unable to extend the resultant aggregates into full cord structures (Fellows, 1994). This may reflect differences between the abilities of homokaryons and dikaryons to redistribute resources through the mycelium, possibly related in part to their different hyphal ultrastructure as previously discussed. The many differences between dikaryon and homokaryon mycelia suggest that they are carrying out very different roles in the environment. The function of the basidiospore-derived homokaryon would seem to be that of dispersal and establishment rather than the persistence and exploitation role of the dikaryon. The homokaryon life-cycle phase of *H.fasciculare* therefore acts in an r-selected manner, dependant on rapid exploitation of transient nutrient supplies. Once established, the existence of a homokaryon in the environment is probably brief due to rapid dikaryotisation by other *H.fasciculare* spores or homokaryons. The dikaryon that subsequently emerges

probably uses the established homokaryotic mycelium as a source from which to draw resources to supply inoculum potential for further colonisation; this may be seen as a change of gear into a K-selected mode. This source-sink relationship between homokaryon and emergent dikaryon has been observed in other wood-decay fungi. (Ainsworth and Rayner, 1990)

Large-scale studies of H.fasciculare cord networks

Both the geometry and translocational ability of *H.fasciculare* dikaryon cord networks have been analysed in various field and soil-tray experiments. In a large-scale experiment (Dowson *et al* 1988 I,II) *H.fasciculare* cord systems were inoculated in woodland soil and the establishment and persistence of *H.fasciculare* in competition with several other fungi was followed over a period of 2 years. The early stages of mycelial establishment were found to be dependant on the microclimate conditions, but later patterns of colonisation were dependent on the availability of resources and the relative combative ability of the fungi. *H.fasciculare* was one of the most successful and persistent fungi in the experiment, only being displaced by the mycelia of *Phanerochaete velutina.* In further laboratory-based studies, image-analysis techniques have been used to measure the fractal geometries and biomass distribution of *H.fasciculare* and *P. velutina* cord networks growing into non-sterile soil from differing resource-bases. (Donnelly *et* al 1995, Bolton and Boddy 1993). Measurements of the fractal dimension 'D' were made for networks of both organisms grown in non-sterile soil trays. *H.fasciculare* cord networks had a higher D value than that of *P.velutina,* indicating a relatively shorter-range, denser-coverage foraging

strategy for *H.fasciculare*. Cord geometry in both organisms was also responsive to initial inoculum size, with denser cord networks emerging from larger inocula.

As has been previously discussed, mycelial cord systems are capable of taking resources assimilated in one area and moving them to another as part of colonisation or combative processes. This may be particularly important in the case of limiting nutrients such as nitrogen or phosphorous. In a field study (Wells and Boddy 1995) the patterns of translocation in *H.fasciculare* cord networks was compared to that of *P.impudicus* and *P. velutina* using ^{32}P radiolabelling. This work indicated that phosphorous was translocated through *H.fasciculare* cord networks to colonised resource components, particularly leaf litter and acorn cupules. Although the cord system was active in this process, little ^{32}P was retained within the cords themselves. Colonised litter components may therefore represent sites of nutrient requirement, safe 'depots' for nutrient storage, or both.

Morphological variability amongst H.fasciculare strains

Although no large-scale study has been made, the pigmentation pattern, emergence and formation of hyphal aggregates certainly varies between different natural dikaryotic isolates. The range of appearance of *H.fasciculare* homokaryons, even sib-related isolates, can also be wide. Although 'white cotton wool' is the commonest appearance, homokaryotic cultures can show hyphal aggregation into cord-like patterns, concentric banded growth and various degrees of senescent morphology with pigmentation and reduced aerial mycelium.

H.fasciculare *as a model organism to investigate heterogeneity*

This study was aimed at analysing the adaptive and organisational properties of *H.fasciculare* in the context of the oxidative-stress model of mycelial morphogenesis, as *H.fasciculare* is a good example of a K-selected combative fungus that is well-adapted to the challenges of environmental heterogeneity. *H.fasciculare* also exhibits interesting internal heterogeneities; the most obvious of these take the form of defined switches between organisational pattern both within and between life-cycle stages. Such variation may represent adaptations to a K-selected lifestyle or represent products of the underlying organisational properties of the mycelium. The other main aspect of *H.fasciculare* that invites further investigation is its boundary chemistry, particularly the production of secondary metabolites; these too are amenable to laboratory analysis. The drawbacks presented by *H.fasciculare* as a model organism include the relatively slow growth rate, the dearth of information on its genetics, and the difficulty in completing the life-cycle in the laboratory. Most of these factors were accepted as irreducible consequences of the choice of a K-selected organism. As *H.fasciculare* is widespread and common there is no difficulty obtaining fresh samples from the wild, and there is potential for the long-term study of different individuals and populations. Finally, there is thought that any such common organism merits study due to its success and presumed importance in the ecosystem.

Chapter Two

General Methods

Sources of H.fasciculare *isolates and samples*

Several *Hypholoma fasciculare* fruit bodies were obtained from Mark Ramsdale on the 5th October 1995. These were taken from two separate sites in Ashton Court woods near Bristol, England. The two samples were designated **IAC** and **IIAC** (Ashton Court samples **I** and **II**)

Fruit bodies were also taken from a felled trunk on the Avenue in the University of Bath grounds on the 16th of October 1995. This sample was designated **AV** (Avenue samples).

Three fruit-bodies (cap diam. 4cm) were used from the IAC sample. Five fruit-bodies (cap diam. 1-2.5cm) were used from the IIAC sample. Three fruit-bodies (cap diam. 3-5cm) were used from the AV sample. Basidiospore and stipe tissue samples were taken from all IAC, IIAC and AV fruit bodies with the exception of fruit-body IIAC-8, which was too small for tissue isolation. As each set of fruit-bodies was isolated from a different cluster with shared morphology, it was assumed that each set originated from a different *H.fasciculare* heterokaryon. The procedures used for the isolation of basidiospore and tissue-sample cultures are described in the methods section of Chapter Three.

H.fasciculare fruit-bodies were also collected for HPLC analysis only. Several fruit-bodies were collected by Jonathan Crowe and Mandy Gilbert from four sites in Leigh Woods near Bristol in November 1996.

Sources of other fungal strains

Phlebia radiata cultures were isolated from a fruit-body collected by Mark Ramsdale at Ashton Court Woods. As with the *H fasciculare* samples, single basidiospore isolates were made from the fruit-body (see Chapter Three methods) and their mating-types determined by pairing experiments (see Chapter Four methods). Heterokaryon cultures of *P.radiata* were isolated from the successfully mated strains; one of these (PH-5) was used in interspecific pairing experiments with *H.fasciculare*.

Fungus KB was isolated from a contaminated matrix-plate culture, where it had been observed to produce a very strong pigmented reaction with a *H.fasciculare* dikaryon strain. Subculturing KB gave a white mycelium with a radial growth rate of 1-2cm/day and considerable combative ability against other micro-organisms. Microscopic examination showed KB had septate hyphae without clamp-connections. The aerial mycelium of KB produced sparse arthroconidia. No other identification was attempted.

Preparation of media used for isolation and growth of fungal cultures

The components indicated in table 1 (except novobiocin, where applicable) were added to a 1l screw-top autoclavable bottle. Distilled water was added to a final volume of 1 litre, the medium was then mixed thoroughly and autoclaved at 115°C for 15 minutes. Novobiocin was incorporated into the media using the following procedure:
0.1g of novobiocin was dissolved in 5ml of SDW and sterilised with a syringe filter. This solution was added to the media when it had cooled to 50°C, then mixed by repeated inversion.

Table 1.1 : Media use and composition

Medium	2% Malt Agar	2% Malt Agar + Novobiocin	Water Agar	2% Malt Liquid
Abbreviation	2MA	2MAn	WA	2ML
Use	General-purpose high-nutrient solid medium.	Solid medium for the isolation of axenic fungal cultures.	Low-nutrient agar in matrix plate experiments.	Liquid culture medium.
Components				
Malt Extract	20g/l	20g/l	-	20g/l
Agar	20g/l	20g/l	20g/l	-
Novobiocin	-	0.1g/l	-	-

Dispensing of media

All media were dispensed in a laminar-flow hood surface-sterilised by spraying with 70% ethanol.

9cm unvented plastic Petri-dishes (Solid media) : 20 to 25ml of molten 2MA or 2MAn was poured by hand into each dish.

9cm unvented plastic Petri-dishes (liquid medium) : 20ml of 2ML was dispensed using an autoclaved hand-pump dispenser.

7ml plastic Bijoux: 5ml of molten 2MA was dispensed using an automatic peristaltic pump. The bijoux were left to cool in a tilted rack to give agar slopes.

Matrix Plates: 2ml (approx.) aliquots of WA and 2MA medium were dispensed into matrix plate compartments using an automatic peristaltic pump. The patterns of medium distribution are detailed in the CH 7 methods section.

Growth and maintenance of fungal strains

Standard culturing procedure for fungal strains

Fungal strains were cultured on 2MA medium in 9cm non-vented plastic Petri-dishes. Plates were inoculated by cutting out a 5mm diameter plug of colonised agar from an established culture with a flame-sterilised cork-borer. This plug was then transferred to the fresh media using a flame-sterilised mounted needle. Growth conditions used were darkness at 20°C, although cultures were exposed to room lighting and temperature during examinations of growth and morphology.

Liquid culture of H.fasciculare *strains*

Three 5mm diameter plugs were cut from an established *H.fasciculare* culture and added to 0.3ml of 2M in a sterile 1.5ml plastic centrifuge tube. A sterile round-ended glass rod was then used to mash the agar plugs into a coarse paste. This paste was then transferred by 1ml micropipettor into 20ml of 2M in a 9cm non-vented plastic Petri-dish. The cultures were incubated in the dark at 20°C for 21 days.

Maintenance of fungal stock cultures

Small ($2mm^3$) plugs of colonised agar were transferred to a 2MA slope in plastic screw-cap bijoux. These were incubated until approximately half of each slope was covered in mycelium, then stored in a refrigerator at 4°C until required.

Metabolite extraction and HPLC protocols

Introduction: HPLC as an analysis tool: uses and limitations

High Pressure Liquid Chromatography (HPLC) is a method widely used for the separation and quantification of biomolecules in complex mixtures. As such, it is an important tool for the analysis of secondary metabolite biochemistry in fungi.

The HPLC apparatus consists of a column of densely packed inert material in which the separation is achieved, a precisely controlled solvent pumping system and a sensitive spectrophotometer. The sample is loaded onto the column and a flow of solvent carries the sample components through the packing material. The interactions between the compounds, packing material and solvent lead to different compounds being retained in the column for different periods of time. As each compound in turn emerges from the column it is recorded by the spectrophotometer.

Different solvents, solvent gradients and columns allow a wide range of samples to be analysed, making HPLC a flexible method. The automated control of sample injection and data collection enables a rapid throughput of samples and computerised data analysis.

As a stand-alone technique, HPLC has limitations as an analytical tool. The retention time and peak shape of metabolites provide useful information, but they say

little about the nature or relative quantity of the compounds present. As the light absorbance constants of different compounds vary enormously, the peak sizes of different metabolites cannot be compared quantitatively without the measurement of the absorbance characteristics of each compound involved. Another limitation is encountered when different compounds have overlapping or identical retention times (RTs). This raises the possibility that some HPLC peaks may be a mixture of different compounds, and that peaks with the same RT seen in two different samples may not be the same compound.

Growth of H.fasciculare *isolates for HPLC*

Hypholoma isolates were inoculated and incubated as described in 'standard culturing procedure' (above).

Ethyl acetate metabolite extraction:

From 9cm Petri-dishes : Colonised agar from a single 9cm Petri-dish was cut into squares ($1cm^2$) and transferred to a screw-top culture jar. 20ml of was added to the jar, the lid was secured to prevent solvent evaporation. The jars were incubated for 12-18 hours in darkness at room temperature.

From matrix-plate agar squares : The colonised agar squares in matrix-plate compartments were quartered and transferred to a screw-top culture jar. Ethyl acetate extraction was carried out exactly as described above, except that only 10ml of ethyl acetate was used.

From *H.fasciculare* fruit-bodies : Each set of fresh fruit-bodies was divided stipe from cap and the two components weighed. Each component (stipes or caps) was roughly ground in 20ml of distilled water using a clean pestle and mortar. The resulting paste was added to a screw-top culture jar. 20ml of HPLC-grade ethyl acetate was added to the jars, the lids were secured and the jars incubated for 12-18 hours in darkness at room temperature.

From liquid cultures of *H.fasciculare* mycelium : The mycelial and liquid media phases of the culture were separated by vacuum filtration using filter papers. The filter-paper and mycelium was washed twice with distilled water, then the mycelium was scraped off and placed in a screw-top culture jar. 5ml of the liquid medium filtrate was added to another screw-top culture jar. 10ml of HPLC-grade ethyl acetate was added to both sets of jars. The jars were incubated at room temperature for 12-18 hours in the dark.

Metabolite recovery

The ethyl acetate containing mycelial metabolites was evaporated to dryness using either a freeze-drying process or a rotary evaporator. Equally satisfactory results were obtained with both methods, but freeze-drying proved more suitable for processing large numbers of samples quickly.

Freeze-drying

Ethyl acetate samples were dispensed into 9ml disposable glass tubes with perforated plastic lids. These were loaded into the freeze-dryer rotor and spun under vacuum until dry. On removal from the freeze-dryer the perforated lids were replaced with intact ones. The dried-down metabolites were redissolved in 1ml or 0.5ml of HPLC-grade methanol and stored in glass vials in darkness at 4°C.

Rotary evaporation

Ethyl acetate samples were loaded into 300ml acid-washed spherical flasks. These were then attached to a rotary evaporator and heated to 35°C using a water bath. The flasks were rotated under vacuum until dry and then sealed with plastic film after removal. The dried-down metabolites were redissolved in 1ml of HPLC-grade methanol and stored in glass vials in darkness at 4°C.

HPLC sample preparation

50µl, 100µl or 200µl of metabolite solution was made up to 400µl (for a 50µl HPLC injection loop) or 800µl (for a 100µl HPLC injection loop) using HPLC-grade solvents to give a final acetonitrile/methanol ratio of 1:1. The diluted sample was centrifuged in a small plastic centrifuge tube at 10,000g in a benchtop centrifuge to pellet any precipitates or debris. 300µl of metabolite solution was transferred to a screw-top HPLC sample vial.

HPLC analysis protocol

The HPLC analysis protocol detailed below was based on the method used by Griffiths *et al* for the analysis of *H. fasciculare* metabolites (Griffiths *et al* 1994*b*). This method is identical to the 'first approximation' method recommended for the reverse-phase (RPHPLC) separation of relatively non-ionic biochemicals (Snyder *et al* 1997). A Gilson HPLC machine (comprising 305 pumps (2), 805 manometer, 811B mixer, 231 autoinjector, 401 dilutor, 506C interface and 712 control software based on an IBM 386 PC) was used with a Phase Sep Spherisorb C18 OD52 (25cm x 4.6 cm) column. The elution of metabolites was recorded by a Gilson UV116 spectrophotometric detector set at 210nm. The liquid phase consisted of an acetonitrile/water mix pumped at a constant flow rate of 1ml/minute. Water is a highly polar solvent, whereas acetonitrile (ACN) is a 'strong' non-polar solvent for hydrophobic compounds. The mobile-phase regime is shown in **Figure 1.1**. The solvent mixture started at 0% ACN and moved to 100% ACN in a linear gradient over 20 minutes; the sample was injected approximately 30 seconds after the start of this gradient. From 20 to 30 minutes 100% ACN was run to completely elute any hydrophobic compounds. The system was re-initialised by returning the solvent to 0% ACN in a two-minute linear gradient, then running 0% ACN for a further eight minutes. The total run time was 40 minutes. The gradient used ensured that all compounds, whether polar or non-polar, were removed from the column after sample injection. When using this RPHPLC method the retention time of compounds was inversely proportional to their polarity; hydrophobic compounds were therefore the last to emerge from the column.

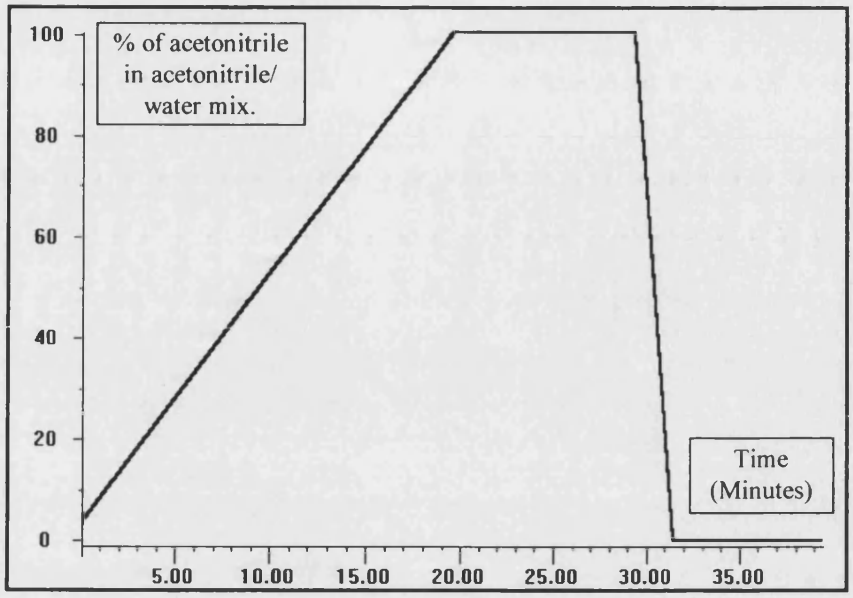


Fig 1.1 HPLC method solvent profile

Data analysis of HPLC results

Gilson 712 software was used for HPLC data analysis. This presented the data in 2 forms; a chromatograph trace showing the change in the 210nm detector voltage, and an integration report produced by computer analysis of the trace. The parameters of the report (section to be analysed, baseline, sensitivity to peak width *etc.*) were chosen to extract the relevant information.

Photography of fungal cultures

Photographs were taken on a copy-stand using a 35mm SLR camera with automatic exposure adjustment and 64 ASA colour slide film. The Petri-plate lids were removed and the plates arranged on a glass sheet supported over a blue-board background. Illumination was by twin halogen lamps; due to this strong lighting, fungal cultures photographed in this manner were not used as stock cultures for subsequent work. Some of the colour slides produced were later digitised using a colour slide scanner.

Chapter Three

Phenotypic variation amongst *H.fasciculare* isolates

Introduction

'Phenotype' in relation to mycelial organisation

The large-scale attributes of phenotype in vegetative fungal mycelia are not simple to quantify; the mycelium has already been described as an adaptable, complex entity with an indeterminate structure. How, then, can a simple 'phenotype' be ascribed to such an organisation?

As mycelial organisation depends strongly on context, much of the answer lies within the control of culture conditions. If these conditions are consistently the same for two fungal cultures, and the cultures maintain a dissimilar appearance, then it is reasonable to say that they have a different phenotype. Under different circumstances the two cultures might look more or less similar to each other, but if a consistent frame of reference is used then meaningful comparisons between fungal strains can be made.

Generation of colony morphology

Many factors contibute to the morphology of a laboratory-grown fungal culture. The most important are due to the phenotype and organisation of the fungal strain in question, but the expression of these is constrained by the culture conditions used. Fungal mycelia can behave like liquids in that they will find the limits of any container they are grown in, and their form will then, at least partially, be defined by that limit.

The morphology of a fungal colony is primarily due to the processes by which hyphae grow and interact with each other during growth, and also to the large-scale movement of resources within the established mycelium. Both of these processes are constrained by the growth conditions chosen. The culture conditions under

consideration here are exclusively batch-culture, that is, growth of the fungi in plates containing a limited supply of unreplenished nutrient. These limitations mean that the mycelium will encounter restrictions both in terms of physical expansion and nutrient exhaustion and staling. Different modes of mycelial growth may react differently to these limits. As an example, rapidly extending cultures may encounter the physical limits of the plate before nutrient exhaustion sets in, giving a relatively evenly distributed mycelium. More slowly extending cultures may exhaust the medium before reaching its limits, giving a mycelial profile with a dense centre and sparse extremities, or in extreme cases, a self-limiting colony which ceases growth.

One of the distinguishing features of many higher fungi is the ability of hyphae to aggregate and interact to form higher-order structures; the greatest development of this potential being found in fruit-bodies and mycelial cords. The interactions of aerial hyphae in a culture is of central importance in determining colony form, leading to varied textures and patterns. Differences in hyphal branching pattern also lead to macroscopic changes in colony morphology, the most striking in *H.fasciculare* being the difference between the frequent, right-angle branching of the homokaryon growth form and the less frequent acute-angle branching of the dikaryon. This fast-effuse/slow-dense dimorphism represents the extremes of hyphal branching pattern, but more subtle differences are often evident even within the same strain.

Sources of phenotypic variation amongst fungal cultures

The origins of colony morphology have been outlined above. Clearly, any factor which can affect any of the processes discussed is a potential source of phenotypic variation. This encompasses the genetic, epigenetic and environmental factors discussed in the

main introduction. Inevitably, fluctuations in experimental conditions make some contribution to variations in the outcome of experiments. Such factors include variable aeration, and temperature regime, light exposure during culture examination and minor changes in the amount or quality of the medium dispensed. These effects can be minimised by comparing only the morphology of cultures grown over the same period of time, in the same incubator and on the same batch of media.

Isolation of basidiomycete strains from the field

The first stage in any microbiological investigation involves gathering strains of the organism for culture in the laboratory. There are several ways to isolate saprotrophic basidiomycete fungi such as *H.fasciculare* from the environment. Vegetative mycelium may be recovered by incubating colonised wood in high humidity. Spores can be trapped from the air using physical traps or biological methods such as exposing homokaryons to spore-bearing air. However, the method most often used is the isolation of strains from fungal fruit-bodies. This method has a number of advantages. Firstly, fruit-bodies are relatively easy to locate and sample. Secondly, the morphology of fruit-bodies provides taxonomic and genetic information about the fungus that is difficult to obtain from undifferentiated vegetative mycelium. Finally, fruit-bodies provide a good source for strain isolation; a concentrated mass of hyphae and spores relatively free from contaminating organisms.

Fruit-bodies provide two main sources for the recovery of fungal strains; basidiospores and somatic hyphae. Isolation of basidiospores from fruit bodies is usually not difficult in readily culturable fungi. Spore prints may be readily obtained simply by leaving the fruit body undisturbed for a few hours over a suitable surface.

This gives a concentrated inoculum of the organism in an easily handled form, which can then be transferred to an agar medium to germinate. The other source of material is the mass of somatic hyhae that make up the structure of the fruit-body, the cap and stipe. Small pieces of tissue may be excised from the fruit body stipe and plated out in attempts to recover the parental heterokaryon. The stipe is more suitable for this procedure than cap tissue as there is less chance of accidently including meiotic tissue. If successful, this procedure will yield the parental heterokaryon of the basidiospores,

Sources of variation in new isolates

Basidiospores may not necessarily be the most representative starting material for further investigations. As most basidiospores are derived meiotically, the cultures grown from them will be recombinant haploid meiotic derivatives from the parental heterokaryon mycelium. As such, they may not reflect ecologically viable genetic or epigenetic combinations even if they give rise to cultures cabable of growth in the laboratory. Only a proportion of the basidiospores from a fruit-body may be capable of completing the life-cycle of the fungus in the wild.

In contrast to basidiospore isolates, the parental heterokaryon is a culture that has genuine 'credentials' in terms of its ability to reproduce and survive in the environment. However, there are problems inherent in regenerating vegetative mycelium from differentiated structures. Fungal fruit-bodies are generally ephemeral by nature; their tissue (excluding the basidiospores) does not naturally participate in any further vegetative growth once it has differentiated from the mycelium. As a consequence of this, there may be no selection pressure to maintain genomic integrity within tissues that do not eventually contribute to the basidiospores. Loss, damage, or

recombination of the fungal genome may occur, possibly even in programmed patterns prompted by the differentiation process. Likewise, epigenetic or extra-nuclear modifications may take place during fruit-body differentiation. Such processes are seen in many tissues in the higher multicellular organisms; well-known examples include the loss of the entire genome in red blood cells and irreversible genetic recombination of the immunoglobin genes in lymphocytes. At least one type of genomic change is known to occur in agaric fruit-body stipe tissue; this involves large increases in the number of nuclei in stipe hyphal compartments and has been recorded in several fungi (Kamada, 1994). *A.bisporus* and *F.velutipes* have been found to contain, respectively, up to 20 and 9 nuclei per stipe cell, whereas up to 66 and 200 nuclei per cell have been found in the stipe cells of *C.cinereus* and *C.radiatus*. This increase in ploidy may be due to the extremely fast extension rate that these stipe cells often exhibit, requiring reorganisations of hyphal scale and structure. It is not easy to predict how cells with such genomic structures might regenerate into a vegetative dikaryon organisation.

Taken in one respect, the subculture of tissue from fruit-bodies simply represents a problem-solving task in recovering an example of the parental heterokaryon. There is, however, another way of viewing the 'problem'. This sees the subculture process as a laboratory in which to study the variability of mycelial phenotypes generated by the disturbances caused by differentiation and regeneration.

Methods

Methods used for isolating *H.fasciculare* cultures

Isolation of single basidiospore cultures

The cap of each fruit body was cut away and placed fertile-surface side down in a sterile, dry petri-dish. Spore-prints were deposited under the caps overnight. The caps were removed and the spore mass suspended in 2ml of sterile distilled water (SDW). The spore suspension obtained was then used as the starting point for a 10-fold dilution series in SDW to an end-point dilution of $1:1x10^{-8}$ of the original suspension.

2MA plates were prepared with a supplement of 0.01% novobiocin to inhibit bacterial growth. Two 200µl replicas of each dilution was spread-plated onto two 2MA plates and then incubated at 20°C in the dark. Germination of the spores was assessed by directly viewing the agar surface of the plates using a light microscope. Most basidiospores germinated after a period of between 1 and 4 days.

After germination had occurred dilution-series spread-plates with suitably spaced germlings were selected for culture isolation. Germlings were identified and marked using a microscope with a low power objective and another dummy objective fitted with a 1mm diameter circular cutting tube. Germlings were centred in the field of view using the low-power objective, then marked out using the flame-sterilised cutting tube. Marked germlings contained in small agar plugs were transferred to 2.5ml of fresh 2MA medium in multi-compartmented culture plates. The transfer was performed under a dissecting microscope at low power using a fine tungsten needle. After 7-10 days of growth at 20°C the colonies were large enough to be subcultured for the establishment of permanent stocks and use in further experiments.

Isolation of fruit-body tissue cultures

Fruit-body tissue was used to provide cultures of the parental heterokaryons. Two small samples (<2mm x 5mm) of tissue were taken from under the surface of each detached fruit-body stipe using a sterile scalpel and tweezers. The samples were transferred to 2MAn plates and incubated in the dark at 20°C. The absence of contaminating basidiospores was confirmed by examination by low-power light-microscope.

Blocks of colonised agar no more than $3mm^3$ in volume were removed from morphologically distinct regions within the mycelial outgrowth using a flame-sterilised scalpel and needle. These blocks were subcultured on 2MA plates to confirm the stability of the various forms of mycelial outgrowth seen on the initial culture plates. The position of the subcultures and the patterns of mycelial outgrowth from the samples were recorded by photography. Stock cultures were made from the subcultures for use in further experiments.

Isolation of conidial cultures from fruit-body tissue plates

The tissue subculture plates were examined and candidate areas for conidial production were identified: these were zones exhibiting sectoring or homokaryon-like growth as opposed to homogeneous dikaryon-like growth. Conidia were obtained by washing the surface of the fruit-body tissue cultures. A 200µl micropipettor fitted with a disposable sterile tip was used to apply 100µl of SDW to the surface of the aerial mycelium bearing conidia. The water was swilled back and forth 4-6 times, then added to 400µl of SDW in a 1.5ml plastic centrifuge tube. The harvest of conidia was assessed by

examining a drop of the conidial suspension under a phase-contrast light microscope. A suitable SDW dilution series of the conidia (giving an end dilution varying from 1:1 to $1:1x10^7$) was prepared and two 200µl replicas of each dilution was spread-plated onto two 2MA plates. The incubation conditions and single-germling isolation procedure was identical to that described above for basidiospore cultures. Conidia germinated after between one and three days of incubation.

Classification of *H.fasciculare* cultures

Growth of colonies for classification

All *H.fasciculare* strains were subcultured and incubated as described in General Methods. Where possible, two replicate plates were made from each strain; this was done with all tissue-plate subculture strains and selected basidiospore isolates but replication was not possible when dealing with the large numbers of conidial isolate cultures.

Classification by life-cycle stage

As described in the introduction, *H.fasciculare* has two distinctive mycelial organisations, the homokaryon and the dikaryon. Homokaryotic mycelium has predominantly right-angle branching, a slow extension rate, lacks clamp-connections and produces oidia from a usually unpigmented aerial mycelium. Dikaryotic mycelium has acutely branching, clamped hyphae, a rapid extension rate and accumulates pigmentation upon aging. Taken as a whole, these characteristics allowed a clear distinction to be made between cultures with homokaryotic and dikaryotic phenotypes.

Classification by colony morphology

Cultures sharing either homokaryotic or dikaryotic phenotypes were further classified into approximate morphological groups using similarities judged by eye. The criteria used included the pigmentation, texture and overall production of aerial mycelium, colony size, and the presence or absence of cords, degenerate mycelium, sectors and banding patterns. Photographs were taken of some cultures; the procedure used for this is described in General Methods.

Results

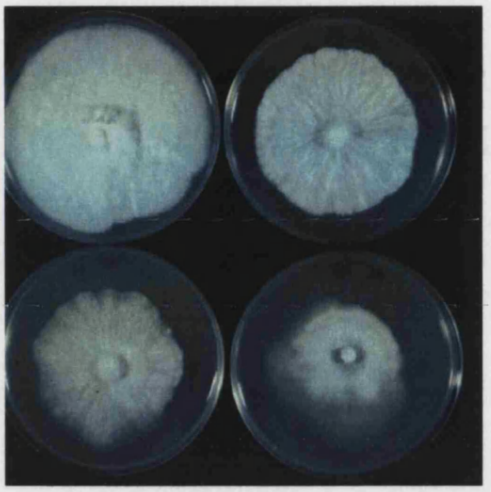
Basidiospore-derived homokaryons: Survival and morphology

A total of 194 homokaryotic basidiospore isolates were recovered from the IAC, IIAC and AV fruit-bodies. The proportion of germlings that survived the transfer process is indicated in ***Table 3.1.*** This was high for both the IAC and IIAC isolates (89% and 96% respectively) but lower for the AV isolates (41%). The surviving basidiospore isolates were subcultured and their morphology studied. The IAC and AV homokaryons showed vigorous, unpigmented growth; in contrast, many of the IIAC isolates showed poor growth with limited aerial mycelium production; several IIAC isolates also showed constitutive orange-brown pigmentation which was not seen in any of the IAC or AV homokaryons. Representative examples of basidiospore-derived homokaryon cultures are shown in **Figure 3.1.** No attempt was made to classify the basidiospore-isolate homokaryons by morphology due to the relatively wide and continuous nature of the variation in their appearance.

Isolation of fruit-body tissue cultures

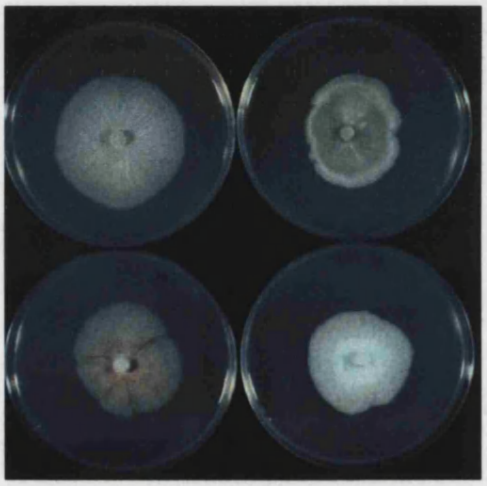
Twenty tissue samples were taken from fruit-body stipes using the procedure described in Chapter 3 Methods. Microscopic examination after inoculation showed no contaminating basidiospores in or around any of the tissue samples. The first signs of hyphal outgrowth from the samples were seen after two to four days. All the tissue samples initially produced a small button of white, fluffy mycelium. In 10 of the cultures, this gave rise to a homogenous outgrowth of dikaryotic mycelium which

Figure 3.1: Morphological variation in homokaryon basidiospore isolates



IAC basidiospore isolates

Strains	13	33
	34	40



IIAC basidiospore isolates

Strains	136	149
	163	152

AV basidiospore isolates

Strains	1	7
	10	13

Table 3.1 : Strain numbering and survival of basidiospore isolates

Fruit body	Spores Trans.	Grew Out	Stock numbers and % survival	Fruit body	Spores Trans	Grew Out	Stock numbers and % survival
IAC-1	*25*	25	AC 1-25	**IIAC-7**	*20*	19	AC 126-145
IAC-2	*25*	21	AC 26-46	**IIAC-8**	*20*	19	AC 146-164
IAC-3	*25*	21	AC 47-67	**Total:**	***100***	**96**	(96%)
Total:	***75***	**67**	(89%)				
				AV-1	*25*	9	AV 23-31
IIAC-4	*25*	23	AC 68-90	**AV-2**	*25*	10	AV 13-22
IIAC-5	*15*	15	AC 91-105	**AV-3**	*25*	12	AV 1-12
IIAC-6	*20*	20	AC 106-125	**Total:**	***75***	**31**	(41%)

Table 3.2 : Summary of outgrowth patterns on tissue-isolate plates

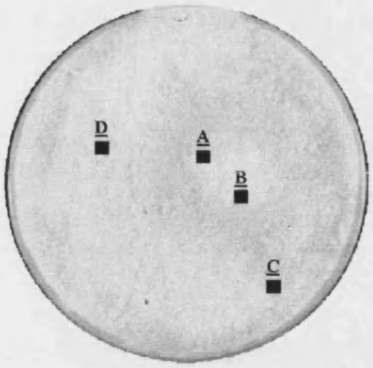
Sample	Sectoring outgrowth	Homogeneous outgrowth
IAC	1a, 1b, 2a	2b, 3a, 3b
IIAC	4b, 6b, 7b	4a, 5a, 5b, 6a, 7a
AV	1b, 2a, 2b, 3a, 3b	1a

rapidly colonised the plates. The other 10 plates showed sectoring of several kinds of mycelial outgrowth. These included fans of dikaryotic mycelium of varied appearance and mycelium with a homokaryotic phenotype. On further incubation the slow-growing, apparently homokaryotic regions were overgrown and surrounded by dikaryotic mycelium, which swiftly colonised the rest of the plate. The entire set is shown, with subculture points, in **Figures 3.3-3.6.**

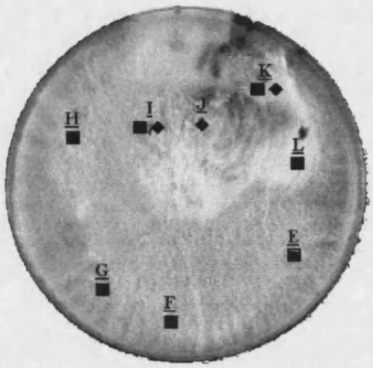
Subcultures from tissue isolation plates

A total of 99 subcultures were taken from the locations shown in **Figures 3.2-3.4**. The majority of subcultures (93) were dikaryons of various appearance; all but 6 of these were morphologically stable and did not sector out into further mycelial regions. The cultures that did show sectoring (such as subculture S7D in **Figure 3.6**) were stabilised by one further round of subculturing (increasing the total number of subs) and did not exhibit any further changes.

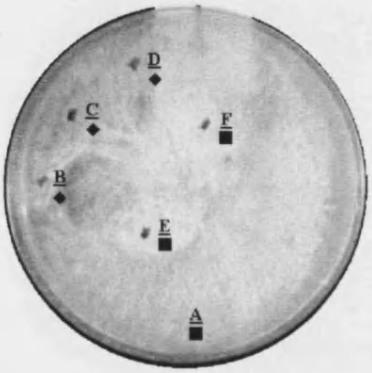
Figure 3.2: Images of the IAC sample stipe tissue regeneration plates, showing mycelial outgrowth patterns and subculture sampling points



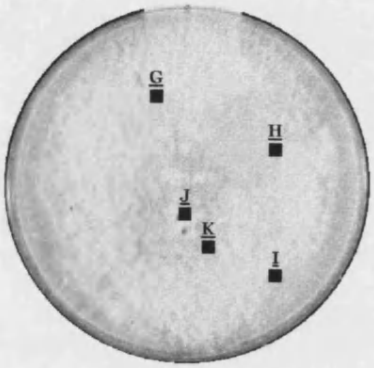
IAC fruit body 1 (a)



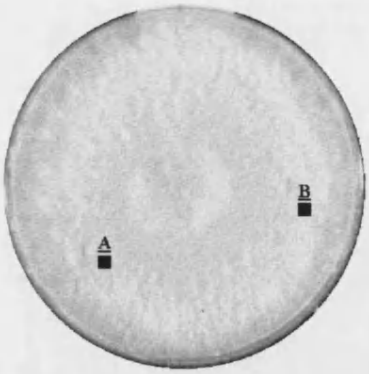
IAC fruit body 1 (b)



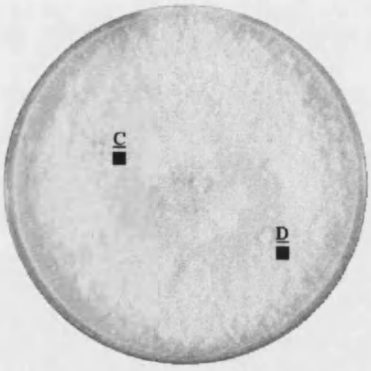
IAC fruit body 2 (a)



IAC fruit body 2 (b)



IAC fruit body 3 (a)

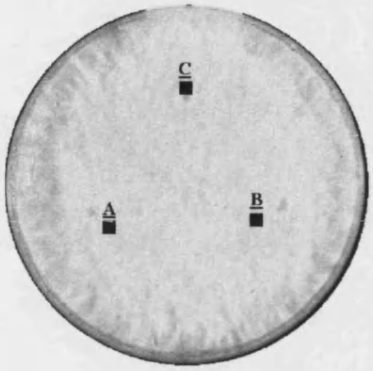


IAC fruit body 3 (b)

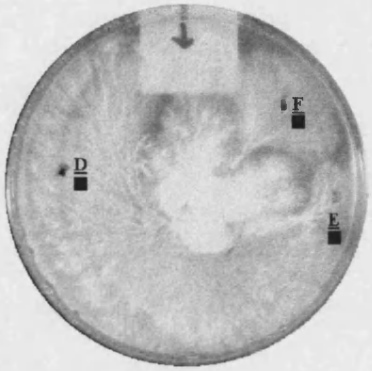
Key to subculture points:

■ = Class 1 dikaryon ◆= Class 2 dikaryon ●= Class 3 (homokaryon)

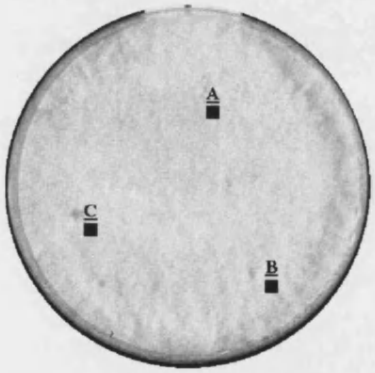
Figure 3.3a : Images of the IIAC sample stipe tissue regeneration plates, showing mycelial outgrowth patterns and subculture sampling points



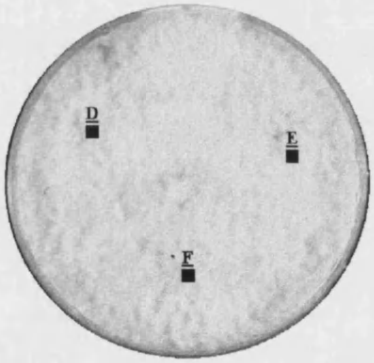
IIAC fruit body 4 (a)



IIAC fruit body 4 (b)



IIAC fruit body 5 (a)

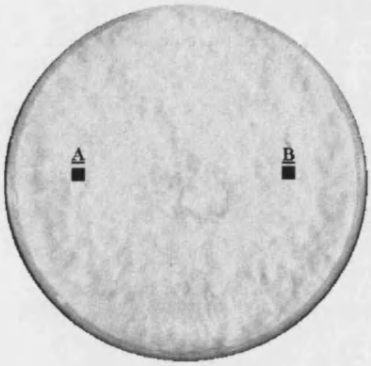


IIAC fruit body 5 (b)

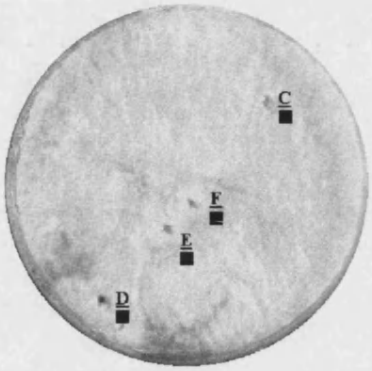
Key to subculture points:

■ = Class 1 dikaryon ◆= Class 2 dikaryon ●= Class 3 (homokaryon)

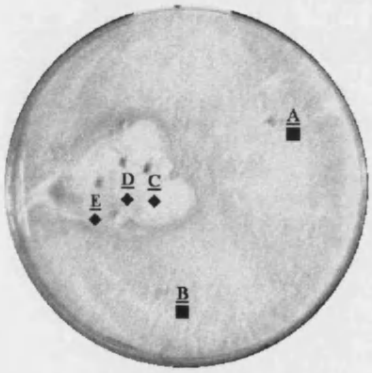
Figure 3.3b : Images of the IIAC sample stipe tissue regeneration plates, showing mycelial outgrowth patterns and subculture sampling points



IIAC fruit body 6 (a)



IIAC fruit body 6 (b)



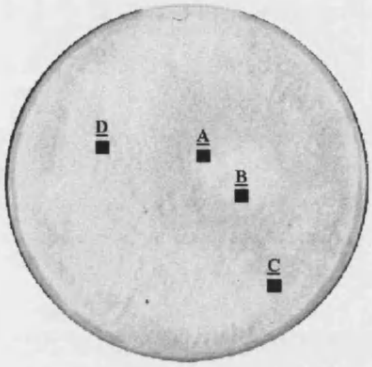
IIAC fruit body 7 (a)

IIAC fruit body 7 (b)

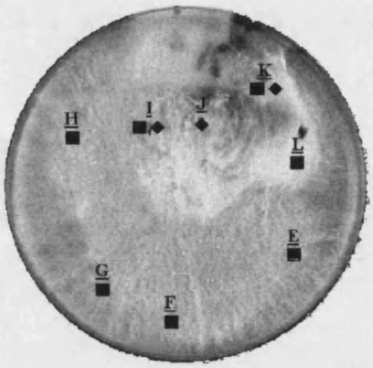
Key to subculture points:

■ = Class 1 dikaryon ◆= Class 2 dikaryon ●= Class 3 (homokaryon)

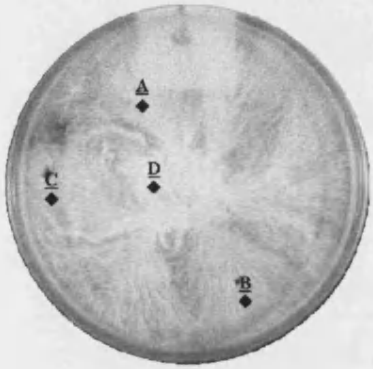
Figure 3.4 : Images of the AV sample stipe tissue regeneration plates, showing mycelial outgrowth patterns and subculture sampling points



AV fruit body 1 (a)

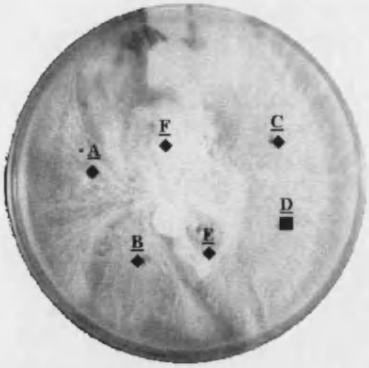


AV fruit body 1 (b)

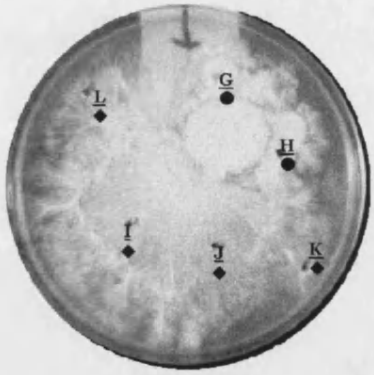


AV fruit body 2 (a)

AV fruit body 2 (b)



AV fruit body 3 (a)



AV fruit body 3 (b)

Key to subculture points:

■ = Class 1 dikaryon ◆= Class 2 dikaryon ●= Class 3 (homokaryon)

Morphology and classification of tissue-plate isolates

The IAC, IIAC and AV tissue subcultures could all be classified into three broad groups termed Class 1 and Class 2 (cultures with a dikaryotic phenotype) and Class 3 (cultures with a homokaryotic phenotype). A description of the criteria used for the classification is provided in ***Table 3.3.*** Subdivisions (1a,1b etc) were made within Classes 1 and 2 according to finer discriminations between phenotypic groups. These sub-classifications were specific to each fruit-body sample set.. Class 3 cultures represented too small a sample for any further subclassification to be made.

Table 3.3 : Classification of tissue subcultures

Classification	Description
Class 1 (59 recovered)	Cultures showing normal dikaryon morphology, including clamped hyphae, rapid (3mm/day) radial growth, thick aerial mycelium and progressive pigmentation.
Class 2 (42 recovered)	Cultures possessing clamped hyphae but growing with appressed or thin aerial mycelium ('Flat' growth) and altered pigmentation patterns. Radial growth rate often reduced to 80-90% of Class 1 culture rate.
Class 3 (5 recovered)	Slow-growing (1-2mm/day) cultures lacking clamp-connections; presumably homokaryotic.

Table 3.4 : Morphology of IAC tissue subcultures

Tissue Plate	Isolate	Class	Tissue Plate	Isolate	Class
S1a	A, B, C	1a	S2a	A, E, F	1b
-	D, E	2	-	B, C, D	2
-	F, H	1b	S2b	G, H, I, J, K	1b
-	G	3			
S1b	I, J, K, L	1b	S3a	A, B	1b
			S3b	C, D	1b

Descriptions of IAC subculture classes

1a) Strong, fast dikaryon. Early emergence of cords within colony, cords extend as colony matures but pigmentation remains minimal.

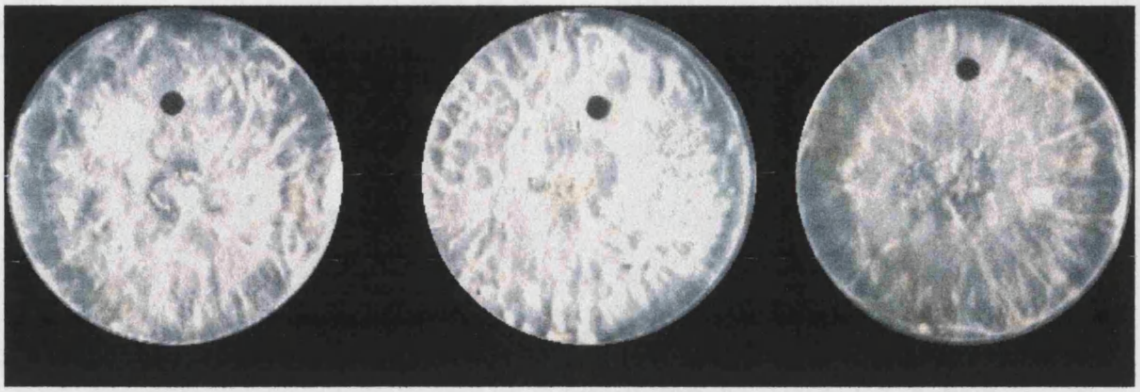
1b) As 1) except cords later-developing or absent.

2) Flat, pigmented growth. Pigmentation accumulates near centre and paler mycelium breaks out at edges.

3) Homokaryon. Smooth, thick growth.

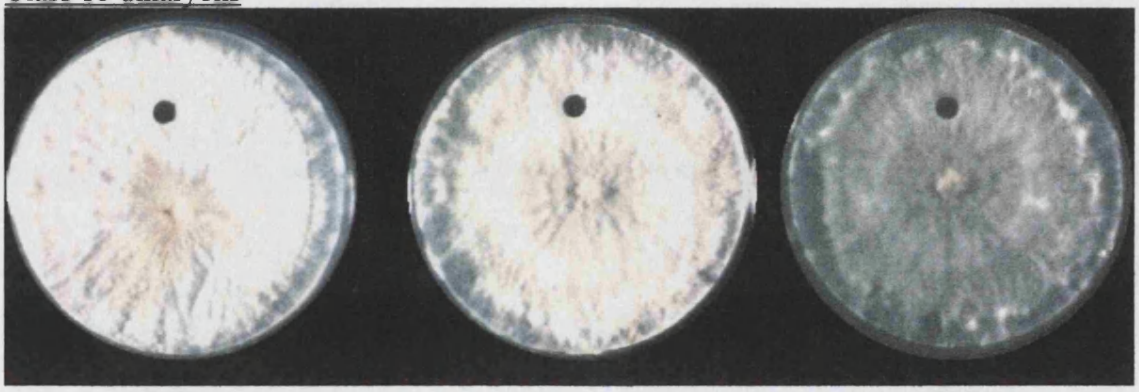
Figure 3.5 : Representative images of IAC tissue-sub dikaryon classes 1a, 1b, 2

Class 1a dikaryons



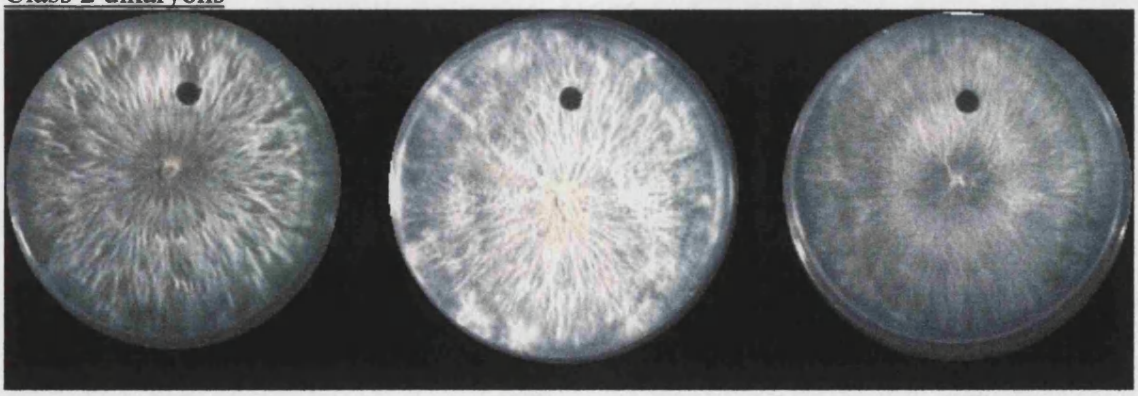
S1A S1B S1C

Class 1b dikaryons



S1F S2A S3C

Class 2 dikaryons



S1D S2B S2D

Table 3.5 Morphology of IIAC tissue subcultures

Tissue Plate	Isolate	Class	Tissue Plate	Isolate	Class
S4a	A, B, C	1	S7a	A, B	1
S4b	D, E, F.	1	-	E, D_1, D_3	2b
			-	C, D_2	2a
S5a	A, B, C	1	S7b	F, G	3a
S5b	D, E, F	1	-	H, I, J, K, I_1, M	2c
			-	L_2	3b
S6a	A, B	1	-	N	2c
S6b	C, D, E, F	1			

Description of IIAC subculture classes

1) Strong, fast dikaryon with powdery yellow pigmentation at centre of plate and coarse cording towards edges. No secondary cord formation on maturation.

2a) Flat, pale dikaryon. No cords.

2b) Flat, powdery yellow dikaryon. No cords

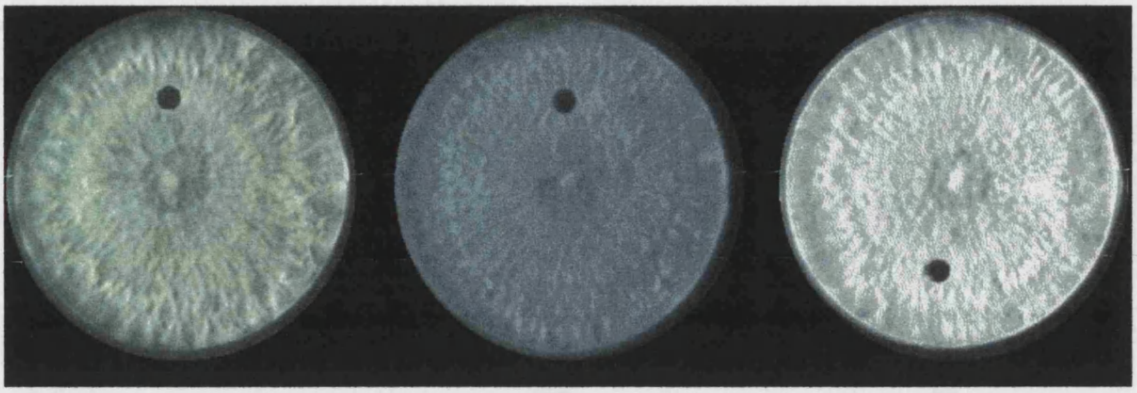
2c) Fast, strong, pale dikaryon

3a) Homokaryon. Occasional thin cord formation within colony and a flat, ragged edge

3b) Uneven, senescent homokaryon with emergent dikaryon sector.

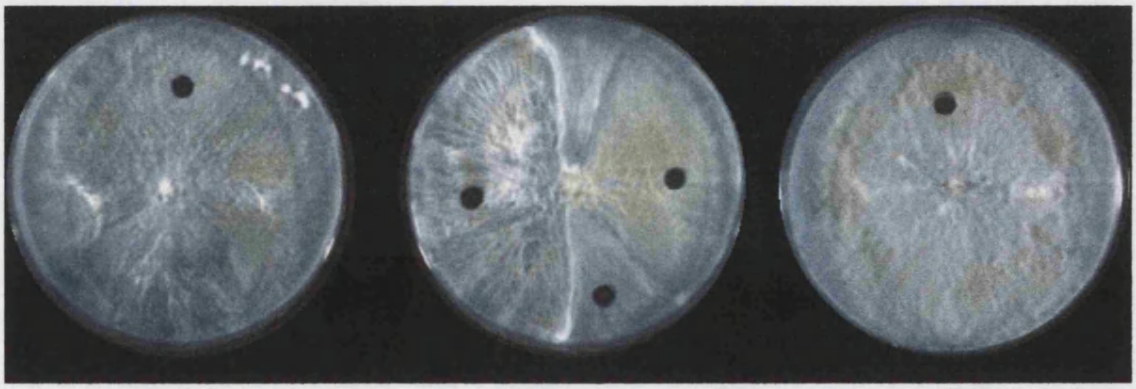
Figure 3.6 : Representative images of IIAC tissue-sub dikaryon classes 1, 2a, 2b, 2c

Class 1 dikaryons



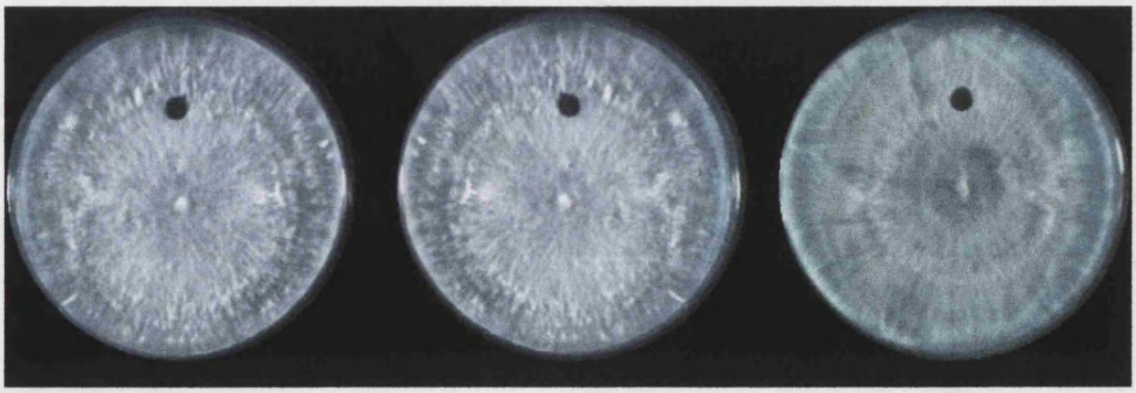
S5A S6E S7A

Class 2a and 2b dikaryons



S7C S7D (class 2b,2a,2b) S7E

Class 2c dikaryons



S7I S7J S7M

Table 3.6 : Morphology of AV tissue subcultures

Tissue Plate	Isolate	Class	Tissue Plate	Isolate	Class
AVS 1a	A, B, C, D	**1**	AVS 3a	A, B, E, F	**2a**
AVS 1b	E, F, G, H, I_1	**1**	-	C, D	**1**
-	I_2, J	**2a**	AVS 3b	G, H	**3**
-	K_1, L	**1**	-	I, J, K, L	**2a**
-	K_2	**2c**			
AVS 2a	A, B, C, D	**2b**			
AVS 2b	E, F, G, H	**2b**			
-	I, J, K, L	**2a**			

Description of AV tissue subculture classes

1) Strong, fast dikaryon maturing into emergent corded orange aerial mycelium. Extensive cording on surface. Fruiting on long incubation

2a) Slow, highly pigmented growth until 1-3cm, then breakout of flat, pale dikaryon at irregular points on colony circumference

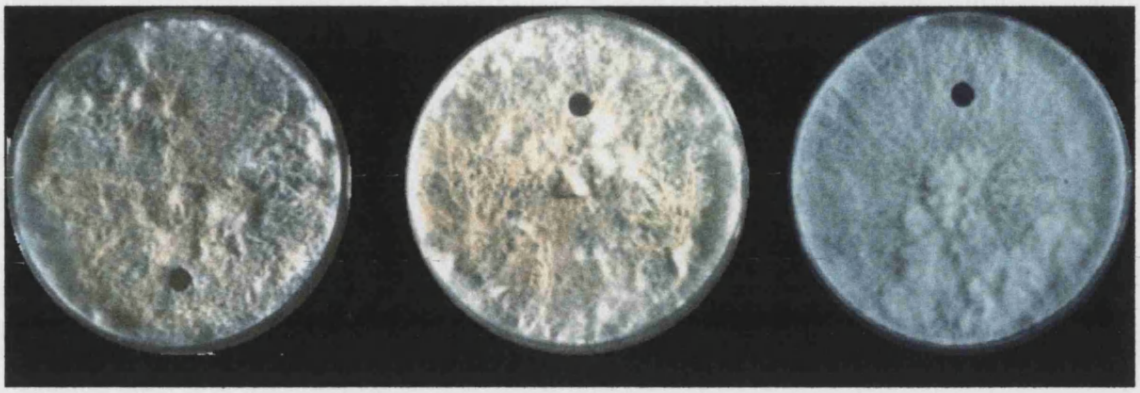
2b) Similar to 2), but paler and with more aerial mycelium.

2c) Flat, pale dikaryon, slower than 1).

3) Homokaryotic, irregular tufts of aerial, occasional banded growth

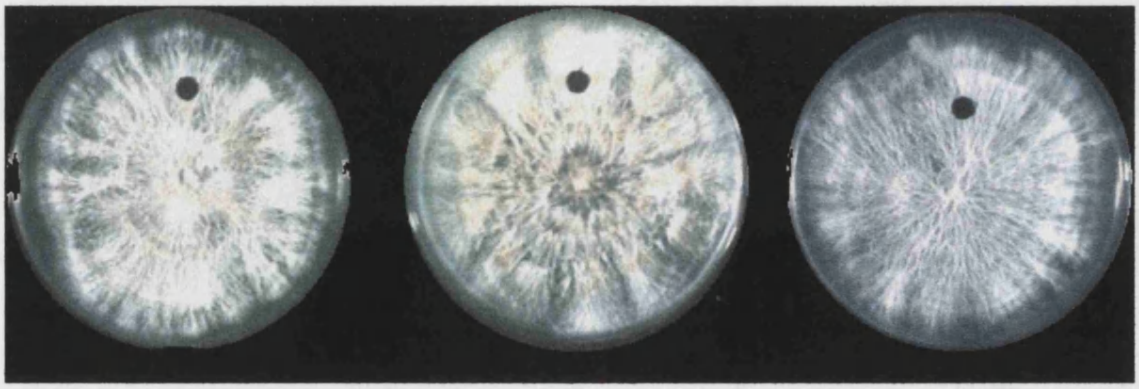
Figure 3.7 : Representative images of AV tissue-sub dikaryon classes 1, 2a, 2b

Class 1 dikaryons



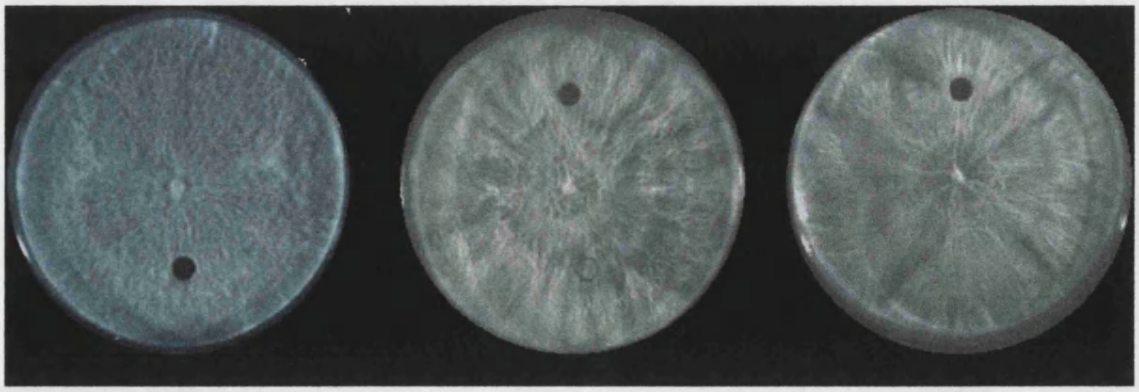
AV1A AV1L AV3D

Class 2a dikaryons



AV1J AV2K AV3F

Class 2c dikaryons



AV2A AV2E AV2H

Isolation of conidial cultures from fruit-body tissue plates

Of the 33 tissue-plate sample areas selected, 22 areas yielded viable conidial cultures. The 480 successfully cultured single-conidial isolates were subcultured to 2MA plates for inspection. 366 (76%) of the single-conidial cultures recovered were phenotypically homokaryotic and 114 (24%) isolates proved to be dikaryotic. Examination of the conidial homokaryon isolate cultures indicated that many groups of isolates shared a similar or identical morphology; it was therefore possible to assign many cultures (but not all) to discrete morphological classes. This was not attempted for the dikaryotic isolates due to the smaller sample size and also in consideration of the fact that such data had already been gathered from the tissue-subculture dikaryons. The morphological classification and numbers of conidial isolates from each set of fruit-body tissue plates area are detailed in ***Tables 3.7-3.9*** below. A more detailed breakdown of the morphological classes recovered from each sample area is included in Chapter 4 results.

Table 3.7: IAC *H.fasciculare* conidial isolate morphology

IAC class	Number of isolates in class	Description of class
1	34	Thick, ridged aerial mycelium
2	2	Flat, ragged edge
3	7	Flat, submerged edges
4	36	Remainder: Variable
D	60	Dikaryons

Table 3.8: IIAC *H.fasciculare* conidial isolate morphology

IIAC class	Number of isolates in class	Description of class
1	7	Thick, irregular ridged aerial mycelium, grows to edge of plate.
2a	45	White smooth aerial mycelium, self-limiting.
2b	6	White smooth aerial mycelium, degenerate margins, pigmented centre.
3	69	Thin, pale aerial. Occasional lobes, sectors, self-limiting.
4	16	Slow initial button of growth, slow, sparse aerial. 0.5 cm width orange ring produced 2cm out from inoculum.
5	11	White aerial with fine cording, self-limiting. Lobed margins.
6	9	Strong growing white aerial. Flat. Occasional irregular cording
7a	23	Slow-growing, self-limiting: Pigmented, submerged mycelium.
7b	6	Extremely slow-growing, colonies (<0.3mm/ day). Irregular, smooth lobes with close banding. Self-limiting.
D	39	Dikaryons

3 cultures not classified

'Self-limiting' colonies were defined as those that ceased growth before encountering the edge of the Petri-plate.

Table 3.9: AV *H.fasciculare* conidial isolate morphology

AV class	Number of isolates in class	Description of class
1	31	Flat, smooth colonies, self-limiting.
2	29	Smooth colonies, growth to edge of plates.
3	26	Strong growth, rippled aerial mycelium. Some droplet formation
4	2	Slow, dense white aerial, irregular
5	3	Irregular sectoring
D	15	Dikaryons

Discussion

Tissue-subculture regeneration

The mycelial outgrowth from regenerated *H.fasciculare* fruit-body stipe tissue gave rise to a variety of mycelial phenotypes *via* subculture and conidial isolation. The cultures exhibiting these phenotypes were stabilised after one further round of subculturing and then maintained this stability through several subsequent subcultures. The patterns of vegetative tissue regeneration were similar in all three of the fruit-body sample sets IAC, IIAC and AV (***Table 3.2,* Figures 3.2-3.4**), suggesting that the generation of cultures of varied phenotype from regenerated stipe tissue is a generic phenomenon rather than being limited to particular *H.fasciculare* individuals.

Variation within dikaryon cultures

The simplest hypothesis of variation in regenerated mycelium from fruit-bodies would predict the recovery of a single type of dikaryon and/or two types of homokaryon. These isolates would represent the nuclei present in the parental mycelium that gave rise to the fruit body. The results obtained here, and summarised in ***Table 3.3*** , present a different and more complicated scenario. Direct transfer subcultures from the tissue plates yielded major classes of normal dikaryons (Class 1), 'flat' dikaryons (Class 2) and a minority of homokaryons (Class 3); further subdivisions were also evident within these classifications. Although the parental nuclei may be present within these varied classes, there are obviously events that also lead to the proliferation of non-parental mycelial phenotypes.

These may involve nuclear or cytoplasmic changes before or during the regeneration process.

Although the majority of conidia recovered from the surface of the tissue outgrowth were homokaryotic, most of the cultures recovered by subculturing were non-conidiating dikaryons, even when subcultures were taken from apparently homokaryotic regions. This disparity is perhaps due to the invasion of dikaryotic hyphae into the conidiating homokaryotic regions. Alternatively (or additionally) some of the 'homokaryotic' regions of growth could have been conidiating heterokaryotic mycelium. The 24% of dikaryotic conidial cultures may therefore indicate the production of mixed populations of homokaryotic/ heterokaryotic conidia from heterokaryotic, non-dikaryon mycelium. Subcultures of such mycelium would be likely to revert to dikaryotic morphology, explaining the disparity between conidia and subcultures. However, it is also possible that these dikaryotic conidial cultures were derived from fragments of dikaryotic mycelia or germlings that had succeeded in mating on the spread-plates prior to isolation.

Variation between basidiospore and conidial homokaryon cultures

Homokaryons were isolated both from basidiospores and from conidia recovered from the tissue-sample outgrowth. The simplest hypothesis of morphological variation in these cultures would be for basidiospore-derived isolates to show a continuous range of variation (due to meiotic recombination) and for the conidia-derived homokaryons to show a two-class distribution of morphology, each of the classes representing one of the intact component nuclei of the parental dikaryon. The distribution of morphology within

the basidiospore-derived isolates matched with this expectation, showing a reasonable approximation to a continuously variable distribution. However, the colony morphologies of the conidial homokaryon isolates did not match the two-class expection. Although many of the conidial cultures fell into discrete morphological classes, there were more of these classes than the two expected (**Tables 3.7-3.10**). This leaves open the possibility that *H.fasciculare* conidia are naturally highly variable due to epigenetic effects. However, this possibility may be discounted based on the observation that single conidia isolated from basidiospore-derived homokaryons germinated into colonies with identical morphology to their parent colony (data not shown). The variations within the conidial isolates therefore present further evidence that there are processes operating, whether genetic or epigenetic, that generate stable variations amongst tissue-outgrowth derived cultures.

Given that such variations occur, the presence of multiple, discrete classes of morphology within the conidial cultures may be clarified if the origin of conidia is compared to that of basidiospores. Each meiotic division that gives rise to a set of four basidiospores involves a relatively random process of genetic reassortment; this gives a 1:1 ratio between the numbers of recombination events and spores. In contrast, the tissue-sample outgrowth may represent a mosaic of mycelial patches with different (epi)genetic structures. Even if these patches are small, each of them may generate a few tens or hundreds of conidia. The ratio between recombination events and conidia is therefore 1: many and the recovery of discrete classes of conidial morphology reflects this; each class may be an asexual group of progeny from a particular mycelial patch.

Variation between the IAC, IIAC and AV samples

The three different sources of *H.fasciculare* used here showed similar patterns of regeneration into broadly similar dikaryon classes. The most striking difference was seen between the homokaryons derived from the IIAC sample and those derived from the IAC and AV samples. A large number of the IIAC conidial and basidiospore isolates showed senescent morphologies characterised by extremely poor growth, limited or absent aerial mycelium and orange-brown pigment production.

The origins of variation in fruit-body tissue isolates

The fruit-body tissue of fungi does not naturally participate in any further vegetative growth once it has differentiated from vegetative tissue. It is therefore the case that reversing this differentiation event by culturing the tissue back into a vegetative mode is a disruptive event not normally experienced within the fungal life-cycle. This may bear comparison with the phenomenon of somaclonal variation in plants. Plant cells from meristems, or even differentiated structures such as leaves, can be grown in tissue culture as undifferentiated callus tissue. This can then be induced to form embryonic tissue and eventually whole plants (somaclones). This process has been found to introduce significant levels of stable phenotypic variation into the somaclone population; further investigations have found a variety of causes including karyotypic, genetic and epigenetic changes (Karp, 1989). Somaclonal variation has been used as a way of generating useful variants of crop plants that are not amenable to traditional plant breeding strategies. Although the gap between plants and fungi is wide, there is evidence that processes similar to somaclonal

variation may occur in fungi during protoplasting. This technique has been widely used in mycology as part of the procedure for genetic transformation using DNA technology. Protoplasting involves the enzymic removal of the fungal cell wall within an osmotically buffered medium, leaving membrane-bound fungal cells (protoplasts) which may then be regenerated into normal fungal colonies. Some studies have found that the protoplasting procedure alone is capable of causing genetic and possibly epigenetic alterations in fungal strains and, as with somaclonal variation in plants, attempts have been made to select 'improved' commercial strains using this method. (Magae *et al* 1988). Quite why protoplasting should generate these effects is not clear, but the wholesale disruption of normal cell structure (including the cytoskeleton; see below) probably has a large impact on many processes that can lead to recombination and epimutation.

If fruit-body tissue regeneration is a process comparable to somacloning or protoplasting, then there are grounds for believing that the emergent mycelial growth will be in a disordered, disorganised state at both genetic and epigenetic levels. A possible manifestation of this disorganisation is the breakdown of dikaryosis in many of the mycelial zones emerging from *H.fasciculare* stipe tissue. This breakdown was revealed by the recovery of homokaryotic conidia from the surface of these regions, which often had a mycelial phenotype closer to the homokaryon than the dikaryon and may be either true homokaryons or transient, disordered (non-dikaryotic) heterokaryons. Similar breakdowns in basidiomycete dikaryosis have been seen in the aerial mycelia of *Schizophyllum commune* and *Flammulina velutipes* (Wessels, 1995, Kemp, 1980) and in an arginine-requiring mutant of *Coprinus congregatus* (Ross *et al* 1991). These observations have

indicated that gene expression is determined not only by the presence of different nuclei in the same cytoplasm, but also by their degree of association. Wessels *et al* (1995) found that aerial heterokaryotic hyphae had altered gene expression patterns compared to the submerged heterokaryotic hyphae of the same mycelium and this correlated with the disrupted, unpaired association of the nuclei in the aerial hyphae compared to the submerged hyphae.

Cytological studies have shown that the cytoskeleton is responsible for maintaining the paired arrangement of dikaryotic nuclei (Kamada *et al* 1993), and disruptions of the cytoskeletal system can lead to the kind of dedikaryotisation effects discussed above. As the cytoskeleton is under a degree of genetic control, and gene expression patterns change with nuclear arrangement, there is scope for interesting positive feedback and hysteresis effects within this system. As an example, a certain level of cellular stress may cause dikaryons to break up, but stress levels below this may be needed for them to re-assemble.

Another arena in which positive feedback effects may be important is that of genomic conflict. Part of the oxidative stress theory of mycelial morphogenesis described in the General Introduction is concerned with the possibility that genomic conflict, resulting in oxidative stress, may result from the combination of different genomes in the same cytoplasm (Ramsdale and Rayner, 1994). One potential way for genomic conflict to manifest itself is by clashes of genetic control over mitochondrial systems, leading to mitochondrial dysfunction and increased oxidative stress. The 'genomic conflict' theory is particularly applicable to the heterokaryotic states of fungi, where two discrete nuclear genomes inhabit the same cytoplasm. One of the consequences of this theory is that fungal

cells under externally induced oxidative stress would have a reduced ability to integrate different, potentially conflicting, nuclear genomes into a functional system. The reduction of integration would introduce further internal oxidative stress and possibly initiate a positive feedback process that could lead to the breakdown of dikaryosis. The occurrence of such breakdowns in the stressed or aerial mycelium of the fungi described above is consistent with this hypothesis. The damage and disorganisation caused in fruit-body tissue during subculturing may create considerable 'external' oxidative stress, and this could be a cause of the morphological and genomic instability evident in the outgrowth from the tissue samples. Once established in a normal growth regime, the oxidative stress-induced variation would be stabilised by the normal coping mechanisms of the mycelium.

Chapter Four

Evidence for a recombinant basis for variation amongst *H.fasciculare* isolates

Introduction

Phenotypic variation; genetic or epigenetic?

The phenotype of an organism is a product of interactions between genetic, epigenetic and environmental input. As the growth of fungal cultures in the laboratory minimises environmental fluctuation, genetic and epigenetic factors are left as the prime determinants of phenotypic variation. The results detailed in Chapter Three showed that significant phenotypic variation was present in cultures derived from stipe tissue outgrowth of *H.fasciculare* mycelium. This variation was greater than that expected from the recovery of purely parental-type genotypes, which would, if epigenetic effects were minimal, have given two types of homokaryon and one type of dikaryon. The purpose of the experiments detailed in this chapter was to find if this phenotypic variation could be attributed, in part or whole, to the presence of recombinant genotypes. If this was not the case, then epigenetic effects would be left as a likely (but currently untestable) candidate for phenotypic variation in these isolates.

Studies of the mating-type of the various *H.fasciculare* homokaryon strains isolated were used to perform a limited genetic analysis that could indicate if any genetic recombination processes were at work during tissue subculturing. The *H.fasciculare* strains used here were all 'wild-type' with no convenient auxotrophic or resistance markers, and a reliable molecular marker system has not yet been established for the species. This left mating-type specificity as the only available genetic marker of recombinatorial processes in *H.fasciculare*.

Mating-type control of development in H.fasciculare

H.fasciculare has a tetrapolar mating system, in which two unlinked loci termed 'A' and 'B' jointly determine mating-type specificity (Fellows, 1994). Only homokaryons different at both loci will mate successfully and form dikaryons. This means that in random crosses 25% of sib-related basidiospore culture pairings will result in dikaryon formation, the remaining 75% being infertile pairings because the homokaryons share at least one A or B allele. Non-sib matings between homokaryons are almost always fertile due to the large number of alleles in the population at both A and B loci. This diversity is due to the recombination of subunits within the complex loci, each sub-locus having several alleles. The net effect of this is an 'outbreeding' ecological strategy (Cooke and Rayner, 1984).

The tetrapolar mating-type system has been genetically dissected in the basidiomycete *Coprinus cinereus*, (Casselton *et al* 1995) and as such systems are often highly conserved between species it is likely that the system in *H.fasciculare* is very similar. The A loci of *C. cinereus* have been sequenced and found to produce homeodomain-related DNA-binding proteins termed *HD1* and *HD2*. Compatible A-locus interactions turn on a cascade of gene transcription which results in unfused clamp-connections, the suppression of conidiation and the ability to form fruit body initials. The B loci of *C.cinereus* have been cloned and are currently being analysed; B-locus function has, however, been characterised in the hemibasidiomycete *Ustilago maydis* (Banuett and Herskowitz, 1994). In this case the B-loci, as in the A-locus system, seem to be a multiallelic non-self recognition system that works on a pheremone-receptor basis instead of homeodomain-dimerisation. Compatible B-loci are required for the clamp

fusion process and the maintenance of stable heterokaryons. In *Schizophyllum commune* it has been found that A=B≠ heterokaryons have a 'flat' phenotype and uncoupled respiration associated with altered mitochondrial function (Hoffman and Raper, 1972.)

Sources of genetic variation

Genetic recombination during meiosis is a powerful source of phenotypic diversity, so it might be expected that meiotically-derived basidiospore isolates would be relatively heterogeneous. This is indeed the case, and in the last chapter homokaryotic basidiospore isolates from *H.fasciculare* were shown to have a wide range of phenotypes. As meiosis is considered to reset most epigenetic modifications to the genome (Jablonka and Lamb, 1995), it is likely that the morphological variation seen in basidiospore isolates is mainly due to genetic recombination. The potential sources of genetic variation within vegetative tissue are less predictable than meiosis; these include parasexual processes and other forms of somatic recombination as discussed in the General Introduction.

Sources of epigenetic variation

Epigenetic modifications have been shown to occur in the vegetative mycelia of fungi, as discussed in the General Introduction. The oidia produced by *H.fasciculare* are conidia formed by a simple process of mitois and hyphal segmentation; they have not undergone the potentially imprint-erasing process of meiosis. With this in mind, it can be appreciated that the potential for epigenetic alterations is greater in homokaryon cultures derived from conidia than those derived from basidiospores. Such alterations

might include the silencing of genes responsible for genomic conflict processes and the activation of genes involved in dikaryon-specific functions, as has been found in *Heterobasidion annosum* (Rayner *et al,* 1995). If all the tissue-derived conidia possessed parental mating-types, then this would present circumstantial evidence of a greater role for epigenetic variations, although the possibility of recombinant processes cannot be ruled out altogether. If recombinant mating-types were recovered from conidial cultures then the disentanglement of epigenetic and genetic variation becomes impossible without the use of more sophisticated tests. In such an event, genetic alterations should probably be considered as the greater contributor to phenotypic variation.

Methods

Experimental determination of mating type in *H. fasciculare* homokaryons

Method of mating-type determination in homokaryons

The mating types of homokaryon cultures were determined by pairing them against each other and recording the outcome of the mycelial interactions. 5mm diameter plugs were cut from homokaryon cultures using a flame-sterilised cork-borer. These were transferred to 2MA medium in 9cm unvented petri dishes using a flame-sterilised needle; the two inoculum plugs were positioned 2-3cm apart in the centre of each plate. The pairing plates were incubated at 20°C in the dark for a period of up to eight weeks. After confluence of the colonies (usually after two to three weeks) the plates were inspected regularly for signs of mating reactions. Confirmatory microscopic examinations of the plates were carried out with a light microscope using a long focal-length, low-power objective.

Design of mating-type experiments for sibling basidiospore isolates

Each pairing experiment included up to fifteen isolates paired with each other in an 'all *versus* all' matrix arrangement. 'Self' pairings of each isolate were included as negative controls, and one basidiospore isolate from an unrelated fruit-body was included in all mating experiments as a positive control. A non-sib-related basidiospore isolate would be expected to be interfertile with all the other basidiospore isolates regardless of their mating-type.

Design of mating-type experiments for conidial isolate homokaryons

The mating-type of the conidial homokaryon isolates was determined by pairing them against basidiospore isolate 'tester strains' of known mating-type. One tester strain was used from each basidiospore isolate mating-type class (See **table**). Selected conidial isolates were paired against all the tester strains and self-paired as a negative control. Due to the large numbers of conidial isolates it was not possible to test them all or to carry out positive controls (non-sib pairings). In addition, some of the more senescent cultures (particularly from the IIAC sample set) did not produce enough mycelium for mating tests to be performed.

***Table 4.1*: Basidiospore isolate tester strains used to analyse conidial isolate MT's**

Mating Type	IAC Testers	IIAC Testers	AV Testers
1	AC-47	AC-84	AV-6
2	AC-13	None recovered	AV-7
3	AC-63	None recovered	AV-10
4	AC-34	AC-100	AV-14

Scoring of mating-type reactions in H. fasciculare *pairings*

Most positive mating reactions between homokaryons could be seen after three weeks. In many cases the reaction would be visible due to the emergence of recognisable tufts and fans of dikaryotic mycelium from the reaction interface. Confirmation was obtained by microscopic examination of the suspected dikaryotic hyphae for clamp-connections, a usually reliable sign that dikaryosis has been achieved.

Another confirmatory test was the appearance of the negative control pairing plates. The A and B mating-type alleles perform different functions in the tetrapolar sexual system, (Casselton, 1995) and observation of the reaction between homokaryons can give hints as to which set of loci is active. Presumed A≠ B= pairing reactions commonly gave a narrow (2-5mm), dense white lens-shaped zone of mycelium where the two cultures met, often containing pseudoclamped hyphae. Presumed A= B≠ pairings often had zones of degenerate and submerged mycelium between the original homokaryon cultures. These zones were wedge-shaped, narrow at the first points of contact between the colonies and widening towards the edge (1-2mm widening to <1cm). It is possible that these zones represent areas of limited nuclear migration followed by the degeneration of unstable A= B≠ heterokaryons.

As with many seemingly simple tests, mating-type pairing reactions produced some results that were difficult to interpret. Firstly, if the homokaryon cultures covered the whole plate, there was no space for readily identifiable dikaryon to emerge. Subcultures from the plate onto fresh medium should have shown emergence of the dikaryon. However, *Hypholoma* matings occasionally failed to dikaryotise the whole of the original homokaryon cultures, so subculturing occasionally gave false negatives. Secondly, false clamp connections sometimes formed in the reaction zone between colonies in presumed A≠ B= mating reactions. False clamps are those in which the hook cell does not fuse with the progenitor hypha. Careful examination usually revealed this, but errors were possible. Subculturing from A≠ B= zones to fresh medium usually yielded homokaryotic cultures, but sometimes a presumed A≠ B=

heterokaryon emerged. These could be distinguished from normal dikaryons by their slower growth rate and almost total lack of pigmentation. They often dissociated into homokaryons upon further growth or subculturing.

Assessment of positive mating reactions was therefore made on the following criteria:

1) Visible emergence of rapidly-growing dikaryotic mycelium.

2) Presence of clamp-connections (self-fused and septate).

3) Visible (A=B≠), (A≠ B=) and (A=B=) reactions with the other three sib mating-types.

Designation of mating-type classes

Cultures were assigned to one of four mating-types depending on the outcome of the pairing experiments. The first basidiospore isolate considered from each set was arbitrarily designated as mating-type 1, the strains then found to mate successfully with this were designated class 4. The remaining two mating-type classes were designated 2 and 3 in the same manner.

Results and Discussion

Patterns of mating interaction in H.fasciculare

The classic model of heterokaryon formation in basidiomycetes involves anastomosis between compatible homokaryons, followed by rapid nuclear migration that swiftly converts both existing homokaryon networks into heterokaryons (Buller, 1931). However, this simple pattern is by no means universal, and exceptions have been demonstrated for the basidiomycetes *Flammulina velutipes* (Ingold, 1991). and *Coniophora puteana* (Ainsworth and Rayner 1990). In the case of *F.velutipes*, anastomosis between homokaryons was infrequent, and the fusion events leading to heterokaryon formation occurred deep within the culture medium. In both of these fungi, dikaryotic hyphae emerged from these central regions and ramified through the homokaryotic mycelium, eventually replacing it due to a combination of higher growth rate and the development of a possible source/sink relationship between the established homokaryons and emergent heterokaryon. There was no evidence of extensive nuclear migration during these mating processes.

Observation of many mating experiments using *H.fasciculare* indicate that it shares many of the characteristics of the *F.velutipes/C. puteana* mating systems, including the dikaryon emergence pattern and limited nuclear migration. Simple subculturing experiments showed that regions of homokaryotic *H.fasciculare* mycelium can persist for several weeks in the presence of dikaryons or compatible homokaryons (Data not shown).

One phenomenon that may represent limited nuclear migration in *H.fasciculare* was evident in A≠ B≠ and presumed A≠B= interactions. Zones of appressed and

senescent mycelium were often observed spreading from the interaction interface around the edges of the participating colonies, but such zones never overtake the whole of the original homokaryotic colony and progressed at a speed not much greater than the growth rate of the colony.

Mating-types of H.fasciculare *basidiospore isolates*

The results of the IAC and AV basidiospore mating experiments show, as expected, four different mating types of basidiospore (***Table 4.2***). However, the IIAC basidiospores tested segregated into only two mating-type groups (designated 1 and 4) after analysis of a total of 23 cultures. Within this sample the Type 1: Type 4 ratio was approximately 1:3. Although the two 'missing' mating types may have been present at low levels, this data indicates that it is unlikely that they follow the normal 1:1:1:1 ratio.

Table 4.2 :

Results of mating type determination of IAC, IIAC and AV basidiospore isolates

Mating Type	**IAC** Basidiospore Isolates
1	8, 55, 47
2	1, 13, 44
3	40, 63, 53
4	17, 19, 31, 33, 34
Outcross	AV-24 Mated with all

Mating Type	**IIAC** Basidiospore Isolates (Pairing 1)
1	69, 152, 161, 84
2	None
3	None
4	75, 89, 94, 98, 102, 107, 116, 122, 124
Outcross	AC-47 Mated with all

Mating Type	**IIAC** Basidiospore Isolates (Pairing 2)
1	149, 103
2	None
3	None
4	97, 100, 106, 136, 163, 120, 92, 140
Outcross	AC-47 Mated with all

Mating Type	**AV** Basidiospore Isolates
1	6, 12, 20
2	7
3	10, 17, 18, 19, 31
4	14, 15, 21, 29
Outcross	AC-63 Mated with all

Key to MT interactions: 1 and 4 are compatible.
2 and 3 are compatible.

Evidence for genetic recombination in conidial isolates

The simplest predictive hypothesis for the distribution of mating-types was that all homokaryons recovered from stipe tissue plates would represent one or other of the parental nuclei. If conidia of just two mating-type specificities were consistently recovered from stipe subcultures, and these two mating-types were compatible, it would be reasonable to presume that these represent the parental nuclear types of the heterokaryon forming the fruit-body. If more than two mating-type specificities were recovered, or if two incompatible mating-types were recovered, then there is evidence

that some kind of genetic rearrangement has occurred either in the fruit-body or during tissue regeneration. The results of the mating-type tests are shown in ***Table 4.3*** and summarised in ***Table 4.4***. These results revealed mixed patterns of recombination and stability depending on both the genetic source and the tissue-plate sample area. These results are discussed below for each genetic source in turn.

Mating-type data for IAC conidia

The IAC conidial sample did not reveal any recombinant genotypes amongst those tested, although only three of nine IAC sample areas yielded conidia and the sample was therefore limited in scope. Homokaryotic conidia of only two mating-type specificities were recovered; these mating types (2 and 3) were compatible, suggesting that they represented the 'escaped' nuclei of the parental dikaryon. A large proportion (43%) of the IAC conidia gave rise to dikaryotic colonies.

Mating-type data for IIAC conidia

The absence of a full complement of basidiospore mating-type tester strains presented an obstacle to the analysis of the conidia from the IIAC tissue subcultures. However, within these imposed limits some results were obtained indicating possible recombination events. Over half (41) of the IIAC conidial isolates tested did not mate with either MT-1 or MT-4 tester strains (the NR group); this could have reflected either a recombinant genome or a more general dysfunctionality evident in the senescent morphology of many of these strains. Those homokaryotic conidia that did exhibit mating reactions included both mating types 1 and 4, but (as in the basidiospore isolates) MT-2 and MT-3 were absent. Although only a few (24) conidia with mating-

types 1 and 4 were recovered, these followed the same approximate ratio seen in the basidiospore isolates, with an excess of MT-4 compared to MT-1. A few (8) conidial cultures with a 'universal' mating type were recovered from two sample areas. These isolates were able to form dikaryons with both MT-1 and MT-4 basidiospore tester strains and present the strongest evidence for recombination events within the IIAC tissue samples. 17% of all recovered conidia gave rise to dikaryotic colonies.

The results of the IIAC basidiospore and conidial mating-type experiments in this chapter provide evidence that the IIAC sample represents a *H.fasciculare* individual with unusual characteristics. Many of the basidiospore and conidial isolates from this source show senescent morphology, although the dikaryon cultures seem relatively normal. The 'missing' basidiospore mating-types 2 and 3, combined with the recovery of parental MT-1 and MT-4 conidial homokaryon strains, indicates that only basidiospores with parental mating-types are produced. This may mean that the meiotic processes in the IIAC individual are dysfunctional. It is also interesting to note that some conidia recovered from the IIAC conidial sample showed a 'universal' mating-type, possessing the ability to mate with both the MT-1 and MT-4 tester strains (***Table 4.3***). Similar universal mating-types have previously been associated with unstable mitochondrial genomes and associated plasmid sequences in strains of *Stereum hirsutum* (Ramsdale and Watkins, unpublished data).

Mating-type data for AV conidia

The conidia from the AV tissue subcultures provided the strongest evidence of somatic recombination. In order to identify the mating-types of the parental nuclei, the assumption was made that these should outnumber recombinant nuclei in somatic

tissue. However, the extent of recombination in this sample was such that the mating-types of the parental nuclei could not be determined. Homokaryotic conidia of all four mating-type specificities were recovered in similar numbers (± 30%) from various sample areas on the subculture plates. In 2 of the 8 sample areas, homokaryons with incompatible mating-types were recovered from the same area. Overall, dikaryons accounted for 10% of the recovered conidia; this represented the lowest proportion of dikaryons recovered out of the three samples.

Table 4.3 : Results of mating-type tests of *H.fasciculare* conidial isolates

Collection Area	MT test results			
IAC	2	3	D	NT
S1a1 (28)	2	22	*3*	1
S1a3 (77)	41	5	*30*	
S1b2 (35)	2	7	*27*	

Collection Area	MT test results				
IIAC	1	4	U	D	NT
S4b1 (23)		4	7	*7*	5
S4b2 (23)	1	2	1	8	11
S4b3 (16)	3	1		*3*	9

Collection Area	MT test result				
IIAC	1	4	D	NR	NT
S7a-1 (15)			*3*	7	5
S7a-2 (15)			*15*		
S7b-1 (22)		3		1	18
S7b-2 (23)		1		1	21
S7b-3 (24)				12	12
S7b-4 (24)				9	15
S7b-5 (26)				11	15
S7b-6 (23)		9	*3*		11

Collection Areas	MT test result					
AV	1	2	3	4	D	NT
AVS 1b1 (10)			5			5
AVS 1b2 (3)					*3*	
AVS 2a1 (14)	1			4	*5*	4
AVS 2a3 (7)	1	3			*1*	
AVS 2b1 (14)	5	8			1	
AVS 3a1 (15)				5	*1*	7
AVS 3b2 (19)	10					9
AVS 3b3 (24)			11			13

Key to column headings

Collection area: Site on the tissue subculture plate from which the conidia were recovered. Next to this, in brackets, is the total number of conidial cultures isolated from that location.

MT test result: Results of the mating-type pairings with basidiospore-derived tester strains. (Key is in box below).

1,2,3,4	Conidial homokaryons with mating type 1, 2, 3 or 4.
NR	Conidial homokaryons with no mating-type response.
U	Conidial homokaryons with 'universal' mating type
D	Dikaryons
NT	Conidial homokaryon isolates not tested for mating type

Table 4.4 : Summary of mating-types present in each sample area

IAC Plates	Sample area	MT of conidia		IIAC Plates	Sample area	MT of conidia
S1a	1	**2, 3, D**		S4b	1	**4, U, D**
	3	**2, 3, D**			2	**1, 4, U, D**
					3	**1, 4, D**
S1b	2	**2, 3, D**				
				S7a	1	**D, NR**
AV Plates					2	**-, D**
AVS 1b	1	**3**				
	2	**-, D**		S7b	1	**4, NR**
					2	**4, NR**
AVS 2a	1	**4, D**			3	**NR**
	3	**1, 2, D**			4	**NR**
					5	**NR**
AVS 2b	1	**1, 2, D**			6	**4, D, NR**
AVS 3a	1	**4, D**				
AVS 3b	2	**1**				
	3	**3**				

Key to mating experiment results	
1,2,3,4	Homokaryons with mating type 1, 2, 3 or 4.
NR	Homokaryons with no mating-type response to any tester.
U	Homokaryons with 'universal' mating type
D	Dikaryon
NT	Homokaryon isolates not tested for mating type

Correlation between conidial homokaryon mating-type and morphological class

The mating-type and morphological class data for conidia from all three genetic sources are compared in ***Table 4.5.*** A simple hypothesis is that, in the absence of recombination, mating type (equivalent to parental nuclear type) should correlate to morphological class. As no recombinant mating-types were recovered from the IAC conidia, this sample should provide the best example of any correlation. However, there is no clear correlation in this sample, with the largest morphological classes (classes 1 and 4) showing, respectively, ratios of 1:2 and 2:1 of mating types 2 and 3. Although these ratios are different, they do not provide particularly strong evidence for morphology/mating-type coupling. Much the same may be said of the results for the IIAC and AV conidia. Although a fit can be seen between mating-type response and morphology in some of these classes (such as between MT-2 and AV class 2, and NR and IIAC class 3) the overall correlation is, at best, weak. Some of the smaller morphological classes (IAC class 3, IIAC classes 5 and 6) do show an identical mating-type within the class, but the small sample sizes involved lend little weight to the observations. The limitations of this experiment lie within the subjective designation of 'morphological class' and the small sample sizes involved. This limits the usefulness of the experiment, especially when genetic recombination and probable epigenetic variations within the sample are taken into account.

Table 4.5 : Mating-type data *vs* morphological class data for conidial isolates

IAC mating types recovered		IAC morphological classes	
2	3	Nº	Total and description
12	22	1	(34) Thick, ridged aerial mycelium
2	-	2	(2) Flat, ragged edge
7	-	3	(7) Flat, submerged edges
23	12	4	(35) Variable morphology (None of above)

AVS mating types recovered					AVS morphological classes	
1	2	3	4	NT	Nº	Total and description
-	2	6	8	15	1	(31) Flat, smooth colonies with appressed submerged margins
2	3	10	1	14	2	(30) Smooth colonies, growth to edge
2	15	-	-	9	3	(26) Strong growth, rippled aerial mycelium. Some droplet formation
-	2	-	-	-	4	(2) Slow, dense white aerial, irregular
1	1		-	-	5	(2) Irregular sectoring

IIAC mating types recovered					IIAC morphological classes	
1	4	U	NR	NT	Nº	Total and description
-	-	7	3	-	1	(10) Thick, irregular ridged aerial, reaches edge of plate.
4	11	1	2	22	2a	(40) White smooth aerial, senescent edge.
-	3	-	3	-	2b	(6) White smooth aerial, senescent edge, pigmented centre.
-	-	-	30	39	3	(69) Thin, pale aerial. Occasional lobes, sectors, senescent edge
				16	4	(16) Slow initial button of growth, slow, sparse aerial. 0.5 cm width orange ring produced 2cm out from inoculum.
-	-	-	6	5	5	(11) White aerial with fine cording. Edge senescent and lobed.
-	6	-	-	3	6	(9) Strong growing white aerial. Flat. Occasional irregular cording
				23	7a	(23) Senescent
				6	7b	(6) Extremely stunted, senescent colonies. Irregular, smooth lobes with close banding.

'Flat' dikaryons and mating-type recombination

There is an interesting possibility inherent in mating-type recombination; this is that recombinant mating-types could lead to the generation of the Class 2 ('flat') dikaryons described in Chapter Three. This possibility is raised by the flat morphology the of A=B≠ heterokaryons of *Schizophyllum commune* (Hoffman and Raper, 1972). Speculatively, nuclei with recombinant mating-types could form viable A=B≠ *H.fasciculare* heterokaryons (or something functionally similar) resulting in the 'flat' Class 2 dikaryons.

The origins of somatic recombination in H.fasciculare *tissue isolates*

Evidence for genetic recombination in regenerated fruit-body tissue has been presented in this chapter. Since this evidence is based on the reassortment of only two loci, only a few conclusions may be drawn from it. There are several techniques that might, in the future, be used to extend the depth and scope of this comparatively simple genetic analysis. Karyotypic analysis via pulsed-field gel electrophoresis could be used to study gross alterations in chromosome structure, while genomic mapping could be carried out using one of the many variant techniques based on restriction-fragment length polymorphisms (RFLPs) or the PCR-based methods such as Randomly Amplified Polymorphic DNA (RAPDs). The finest details could be obtained by the DNA sequencing of regions (such as mating-type genes) known to have undergone recombination.

The first conclusion that can be drawn from the data is that the recombination seen in the conidial isolates is unlikely to be programmed as part of the fruit-body differentiation process. If this was the case, then the the pattern of recombination

products would probably be less variable and irregular between both genetic sources and sampling areas. This leaves the option of relatively random meiotic-like or parasexual processes occurring within the fruit-body or during the regeneration process. Although there may be pre-existing recombination within the fruit-body, there are strong reasons for believing that the stress and disorganisation of regeneration provides the window during which recombination is most likely to occur. Many of the somatic recombination processes described in the General Introduction have been seen to increase in frequency when the fungi in question are exposed to altered or stressful circumstances. As discussed in Chapter Three, the regeneration of vegetative mycelium from fruit-body tissue probably qualifies as a structurally, oxidatively and genetically stressful event and it is therefore perhaps unsurprising that somatic recombination occurs during this process. A comparison of the recombination frequency between *H.fasciculare* mycelium derived from fruit-bodies and colonised wood might be interesting; substrate mycelium within a wood resource unit may have better buffering against oxidative stress than the 'naked' mycelium of a fruit-body.

A significant question for fungal experimentation arises from these findings: Is somatic recombination being taken into account when fruit-body tissue isolation is used as a source of fungal strains for experimental analysis? If not, then a potentially large source of heterogeneity is being fed, unknowingly, into the experimental mill.

Chapter Five

Major patterns of variation in HPLC profiles of *H.fasciculare* mycelium

Introduction

Introduction

In the General Introduction a model of fungal growth and differentiation was presented from which specific predictions could be made about the patterns of metabolite distribution within and between different mycelial organisations of *H.fasciculare*. A variety of such mycelial organisations were isolated from fruit-body tissue and have been described in Chapters Three and Four. This chapter is concerned with the major patterns of metabolite production exhibited by *H.fasciculare* strains representing all the different patterns of mycelial morphology encountered in Chapter Three. An analysis of the metabolite profile of *H.fasciculare* fruit-bodies is also included.

The secondary metabolites of *H.fasciculare*

There is a small but detailed literature concerning the secondary metabolites that have been found in the mycelium and fruit-bodies of *Hypholoma* species. A summary of the metabolites investigated, with references, is supplied in ***Table 5.1.*** These compounds have attracted attention for several reasons. Firstly, secondary metabolites have been used taxonomically to aid in the classification of fungal species. Secondly, compounds from *Hypholoma* species have had interest as toxins, potential antibiotics and drugs. This has led to detailed studies of the chemical structure of the *Hypholoma* caryophyllane and styrylpyrone metabolites. In addition, perhaps as a result of recently increased environmental awareness, there have been surveys of chlorinated organic compounds, of which *Hypholoma* species seem to be major producers.

White-rotting fungi have been found to produce several redox-cycling compounds such as anisyl alcohols as part of their ligninolytic system; these are

metabolites that enable the transfer of H_2O_2 generating power to extracellular lignin and manganese peroxidases. Chlorinated variants of such compounds have been isolated from wood-rotting fungi and it has been hypothesised that the halogenation of such redox-cycling compounds may aid their function by making them more robust in a chemically aggressive extracellular medium. The chlorinated compound identified in Griffith *et al* (1994*b*) may represent the chemical product of a reaction between two such chlorinated anisyl metabolites (CAMs). As lignin degradation is a secondary metabolic process, generating less energy than it consumes, CAMs and their allies may be regarded as secondary metabolites. However, since they are part of a regenerating enzymatic cycle they do not fit directly into the insulative/ protective role envisioned for secondary metabolites in the oxidative stress model of hyphal differentiation.

HPLC and the analysis of fungal metabolites

High Pressure Liquid Chromatography is a powerful method for analysing the often complex mixtures of fungal metabolites extracted from cultures. HPLC involves the separation and subsequent spectroscopic detection of the component metabolites using a chromatographic column and a variable solvent regime. A more detailed description and introduction to the technique is included in Chapter 2, General Methods. HPLC can be used for quantifying the production of known fungal metabolites and identifying new ones. HPLC 'fingerprints' of metabolites produced by different species have been used as a taxonomic aid to fungal classification (Wildman 1995). In addition, the retention time of a metabolite within the HPLC separation column can provide some limited information about its chemical nature.

The boundary chemistry of H.fasciculare: Models and predictions

The model for mycelial growth and differentiation described in Chapter One predicts that mycelial systems will protect themselves from oxidative stress, at least in part, by the production of insulative secondary metabolites. This can tested experimentally by analysing the metabolite production of mycelia and matching metabolite profiles to observable states of mycelial differentiation. Mycelial organisations with the most K-selected properties should be correlated with the production of hydrophobic insulative metabolites.

The present HPLC study was designed to take in all the major life-cycle stages of *H.fasciculare*; the homokaryon, the dikaryon and the fruit body. Several examples of each life-cycle stage were analysed to ascertain if differences between these mycelial organisations were correlated with differences in the pattern of ethyl-acetate extractable metabolites. In addition, strains within the same life-cycle stage with differing morphologies were included for study. The main division here was between Class 1 (normal) dikaryons and Class 2 ('flat') dikaryons isolated from fruit-body tissue. A number of basidiospore-derived homokaryons were also analysed, although the phenotypic differences between sibling homokaryon strains were more complex and continuous than the Class 1/ Class 2 division between dikaryon phenotypes. This meant that any predictions or correlations between phenotype and HPLC profile would be correspondingly more difficult for these strains.

The main prediction that can be made from the oxidative-stress model of metabolite production is that the *H.fasciculare* dikaryotic mycelium (K-selected life-cycle stage) should have a greater hydrophobic metabolite content than the homokaryotic mycelium (r-selected life-cycle stage). These hydrophobic compounds

could represent the insulative material that enables the increased combative and translocative abilities of the dikaryon. Previous HPLC work on *H.fasciculare* metabolite production (Griffiths *et al* 1994 a,b) has outlined important features of the *H.fasciculare* metabolite profile. A summary of the major peaks encountered in this study is provided in ***Table 5.2***.

Table 5.1 Previously identified *H.fasciculare* metabolites and references

Metabolite	Location	Structure and properties	References
Naematolon Naematolin Naematolins B,C,G	Mycelium	Caryophyllane sesquiterpenoids	**Backens *et al* 1984** Ito *et al* 1967 **Doi *et al* 1986, 1990**
Hypholomins A, B Fasciculins A, B bis noryangonine hispidine	Basidiome	Yellow pigments Styrylpyrone Styrylpyrone, yellow pigment	Fiasson *et al* 1977 Gluchoff-Fiasson, Kuhner 1977
CAM	Mycelium, Wood	Chlorinated anisyl metabolites (peroxigens?)	**Field *et al* 1995** **Griffith *et al* 1994**

Table 5.2 H.fasciculare dikaryon HPLC peaks with retention times (RT) and TLC results from Griffith *et al* 1994*b*

HPLC peak	RT (Min)	Analysis of corresponding TLC spot
Ha	4.3	No data
Hb	10.0	tH-5: steroids,sterols,quinones, No napthoquinones (Spot turns red)
Hc	10.8	tH-5: steroids,sterols,quinones, No napthoquinones (Spot turns red)
Hd	11.5	No data
He	14.5	tH-4: sterols,phenols,quinones, napthoquinone
Hf	15.5	tH-4: sterols,phenols,quinones, napthoquinone
Hg	18.9	No data

Methods

Selection of strains for HPLC analysis

Strains of *H.fasciculare* for HPLC analysis were selected from the range of isolates described in Chapter Three. Only a limited number of these strains could be analysed in detail, so a sample of 30 dikaryon strains and 24 homokaryon strains was selected. These strains are detailed in ***Tables 5.3*** and ***5.4***. The dikaryon cultures were selected from the tissue-plate subcultures. Three different classes of morphology were present in these isolates, as has been described in Chapter 3; normal dikaryons (Class 1), 'flat' dikaryons (Class 2) and homokaryons (Class 3). Ten examples of Class 1 and 2 dikaryons were chosen from each of the tissue isolate sets IAC, IIAC and AV for HPLC analysis. Tissue-plate homokaryons (Class 3) were not used due to the small sample size. The homokaryons used for metabolite analysis were all taken from the basidiospore isolate cultures, as these were generally more vigorous than the conidial isolates and represented a greater range of morphology. Eight single-basidiospore cultures were selected from each of the IAC, IIAC and AV spore collections. Fruit-body tissue was also analysed by HPLC, but as *H.fasciculare* fruit bodies were not readily available from laboratory cultures, several were collected from local sites as described in General Methods.

Growth of cultures and extraction of metabolites

H.fasciculare cultures were inoculated onto 9cm 2MA plates and incubated as described in General Methods. Four plates were inoculated with each strain; allowing for two harvests of two replicate plates each. The first harvest was carried out after

two to three weeks, the second after eight to ten weeks. The growth time in days is given in ***Table 5.5***. Metabolite extraction and HPLC analysis was carried out as described in General Methods. The sites where *H.fasciculare* fruit-bodies were found and the metabolite extraction and HPLC procedures used are also described in General Methods.

Analysis of HPLC data

The HPLC results were available in the form of chromatogram traces and computer-analysed peak integration tables (reports) of those traces. Qualitative peak presence/absence tables were compiled by examination of the chromatogram traces and used to identify interesting peaks for quantitative analysis. This was carried out by recording the relevant integrated peak areas from the reports and comparing the data obtained for different harvests, strains and morphological classes. A comparison was made between the results obtained here and published data for *H.fasciculare* HPLC analysis.

Statistical analysis of HPLC metabolite peak data

In the results section of this chapter, 95% confidence limits are used to indicate metabolite peak variability about a mean level. This measurement of 'confidence' is designed for large sample sizes following a normal distribution. It should be emphasised that the sample sizes used here (typically 3-7 samples) are smaller than ideal for this measurement and their distribution pattern unknown. In view of this, the confidence limits should be regarded as an estimate rather than a measurement.

Table 5.3: Dikaryon tissue isolate sets used for HPLC analysis

5.3a

Tissue Plate	Isolate	Morph Class
S1a	A	1a
S1a	B	1a
S1a	D	2
S1a	E	2
S2a	E	1b
S2a	B	2
S2a	D	2
S2b	G	1b
S2b	H	1b
S3b	C	1b

5.3b

Tissue Plate	Isolate	Morph Class
S6b	D	1
S7a	A	1
S7a	B	1
S7a	C	2a
S7a	E	2b
S7b	H	2c
S7b	I	2c
S7b	J	2c
S7b	M	2c
S7b	N	2c

5.3c

Tissue Plate	Isolate	Morph Class
AVS 1b	F	1
AVS 1b	G	1
AVS 1b	I_1	1
AVS 1b	K_2	2c
AVS 2a	C	2b
AVS 2b	L	2a
AVS 3a	C	1
AVS 3a	D	1
AVS 3b	I	2a
AVS 3b	L	2a

Table 5.4: Basidiospore-derived homokaryon isolate sets used for HPLC analysis

5.4a

Fruit Body	Isolate	MT
IAC	13	2
	33	4
	34	4
	40	3
	44	2
	47	1
	55	1
	63	3

5.4b

Fruit Body	Isolate	MT
IIAC	75	4
	94	4
	100	4
	124	4
	136	4
	149	1
	152	1
	152	4

5.4c

Fruit Body	Isolate	MT
AV	1	-
	7	2
	9	-
	10	3
	12	1
	13	-
	23	-
	24	-

Table 5.5: Incubation times for isolate sets used in HPLC analysis

Isolate sets	First harvest	Second harvest
IAC Dikaryons	14 days	68 days
IIAC Dikaryons	20 days	65 days
AVS Dikaryons	17 days	50 days
IAC, IIAC and AVS Homokaryons	14 days	65 days

Results and Discussion

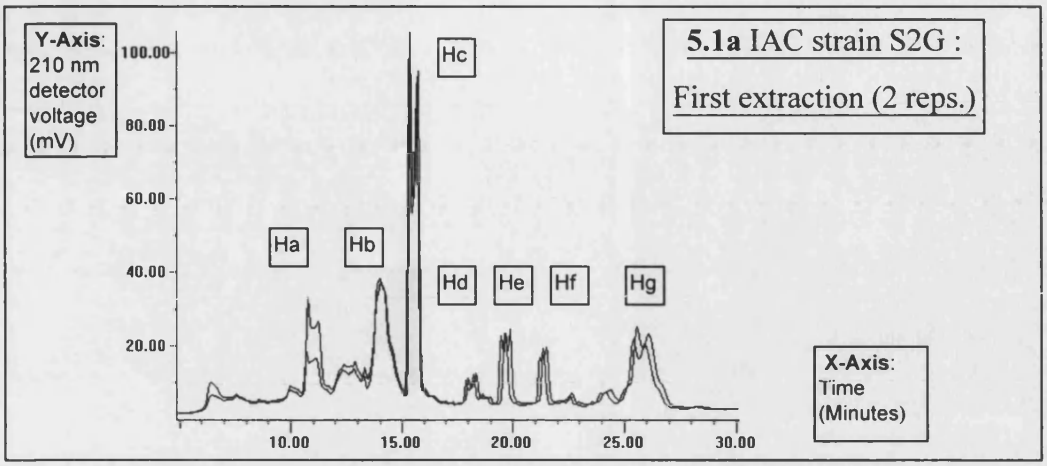
Comparison of HPLC metabolite profiles with published data

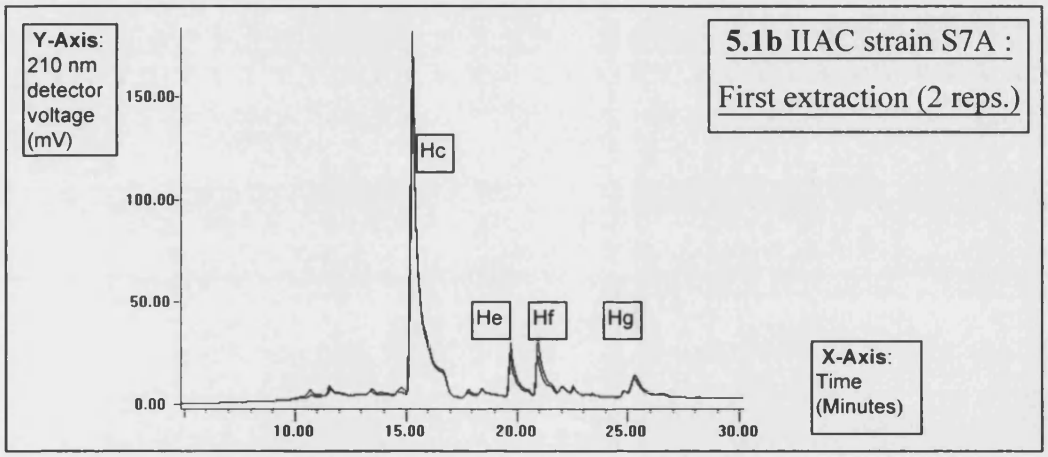
The comparison of the relative peak sizes and retention times enabled an identification to be made between the metabolite peaks in the typical HPLC traces of Class 1 dikaryons shown in **Figure 5.1** and those recorded in ***Table 5.2*** for a *H.fasciculare* dikaryon in Griffith *et al* (1994*b*). Although there were differences between the Class 1 profiles from each of the sources of *H.fasciculare* strains, there were enough features in common for the peaks Ha through Hg to be identified in strains derived from each of the IAC, IIAC and AV fruit-body sets. The HPLC data for Class 2 dikaryons and homokaryons (**Figures 5.2, 5.3**) indicated that the peaks recorded for these cultures were a subset of the Class 1 dikaryon peaks Ha-Hg and did not include any substantial peaks that could not be identified as such. In contrast, the peaks recorded from the HPLC of fruit-body material (**Figure 5.4**) could not clearly identified with peaks Ha-Hg; some overlaps existed but these could not be considered as significant within the limitations of the HPLC method. The fruit-body peaks were therefore given separate identities as Peak Clusters 1, 2 and 3. The comparison of HPLC traces for replicates of each individual *H.fasciculare* isolate in **Figures 5.1-5.3** shows that reproducibility within the experiments was high; this was true for the majority of strains studied.

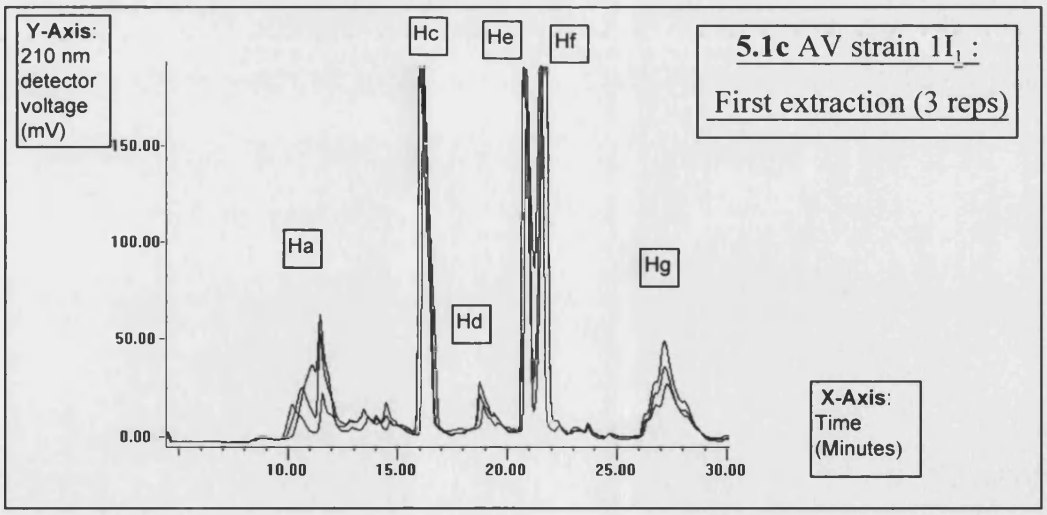
Qualitative features of HPLC traces from H.fasciculare cultures

After the peaks Ha-Hg had been identified, a qualitative survey of peak absence/ presence was carried out for all the *H.fasciculare* isolates. The results are presented in **Figs. 5.5-5.10** and summarised in ***Table 5.6***. These data shows that there are

Figure 5.1 Representative samples of HPLC traces for Class One (normal) dikaryons

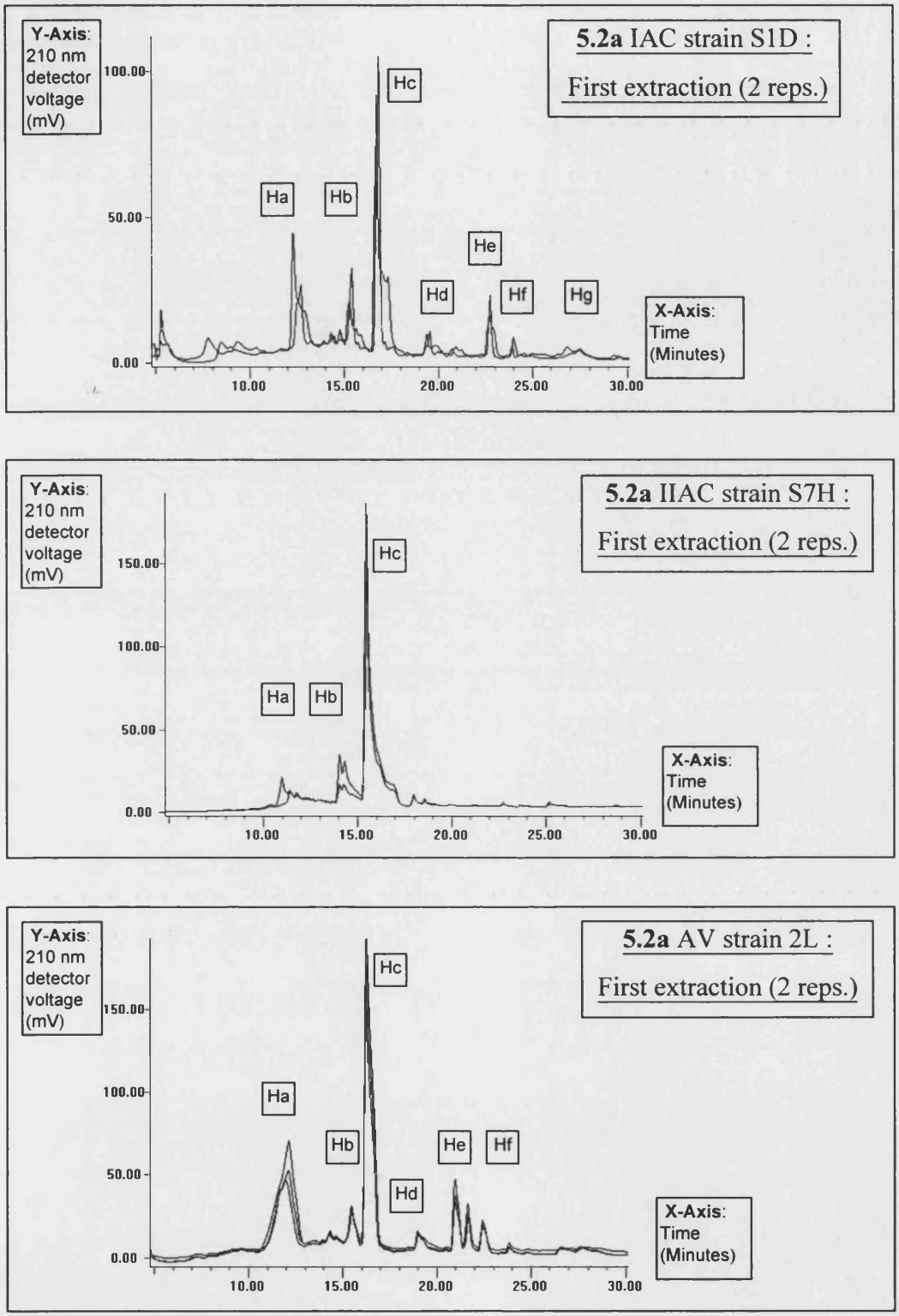






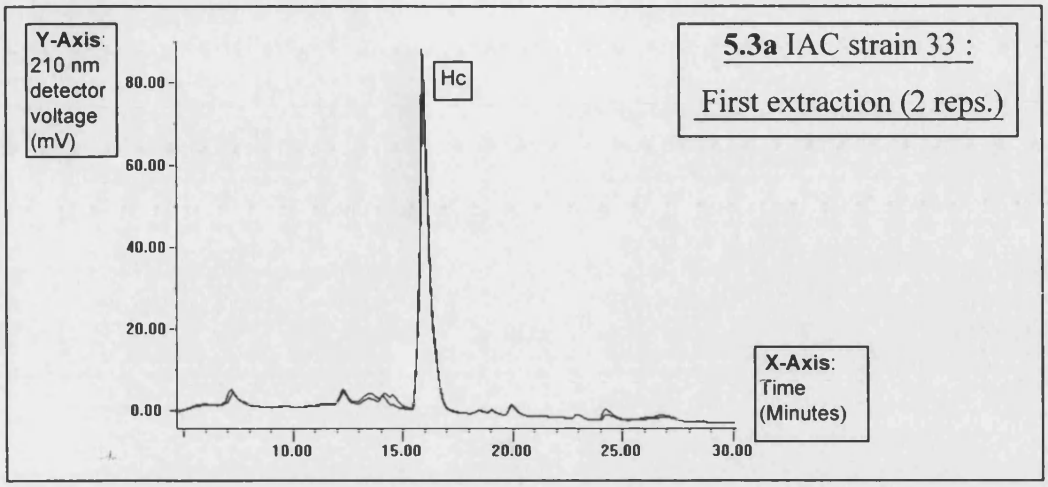
Peak identities (Ha-Hg) allocated according to Griffith *et al* 1994*b*.

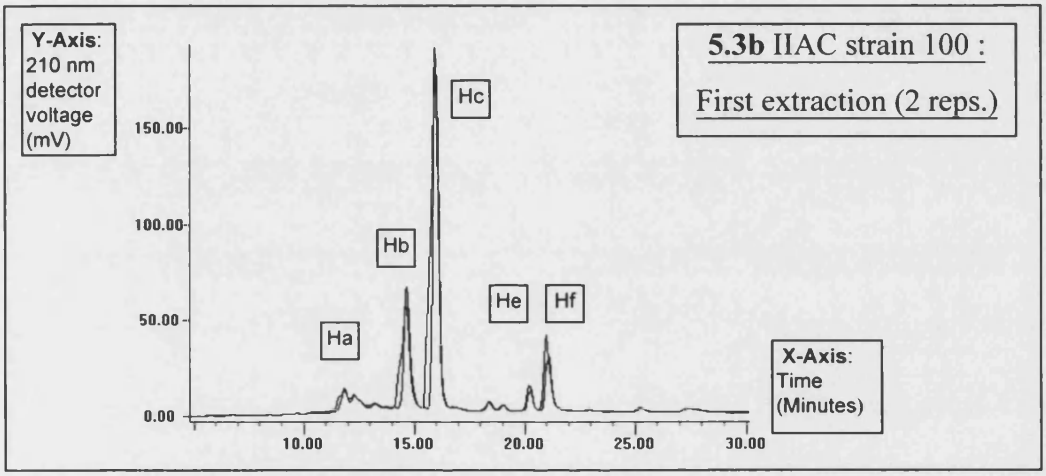
Figure 5.2 Representative samples of HPLC traces for Class Two ('flat') dikaryons

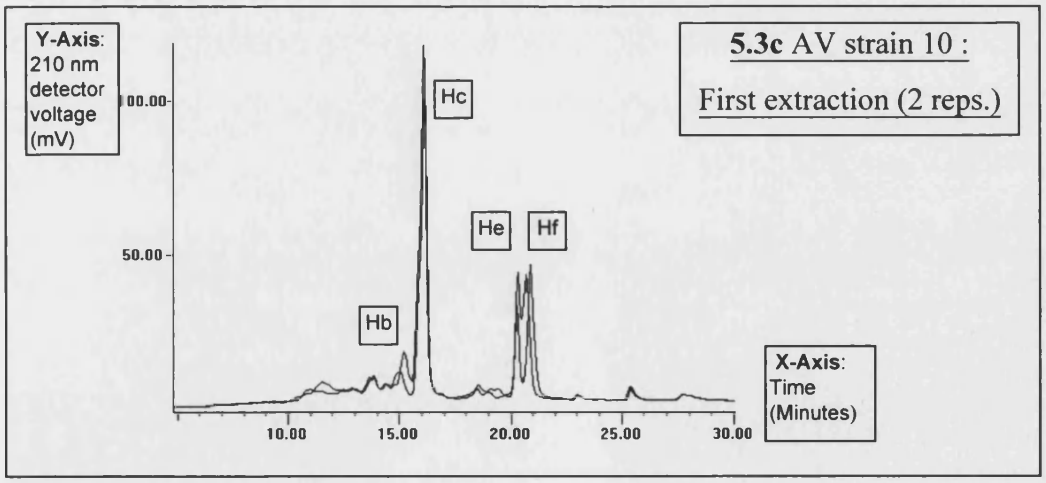


Peak identities (Ha-Hg) allocated according to Griffith *et al* 1994*b*.

Figure 5.3 Representative samples of HPLC traces for homokaryons

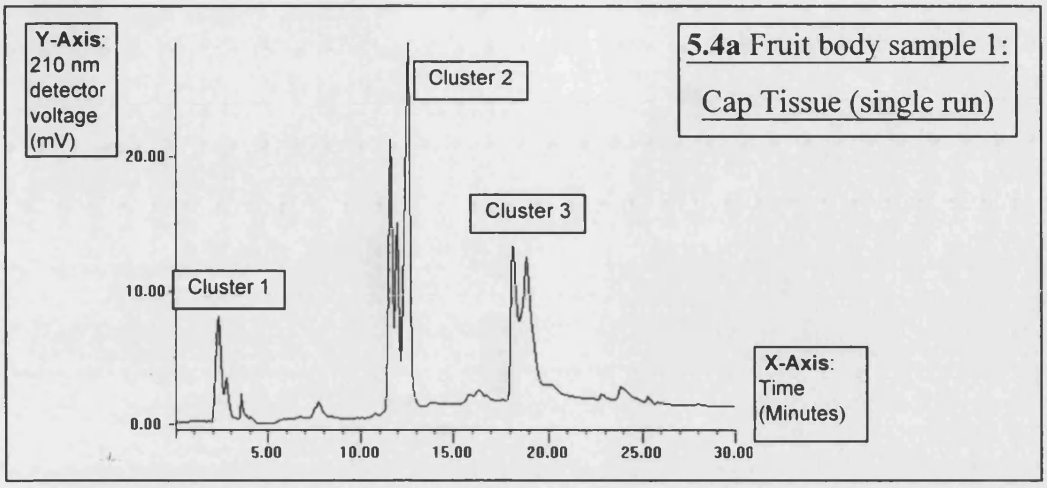


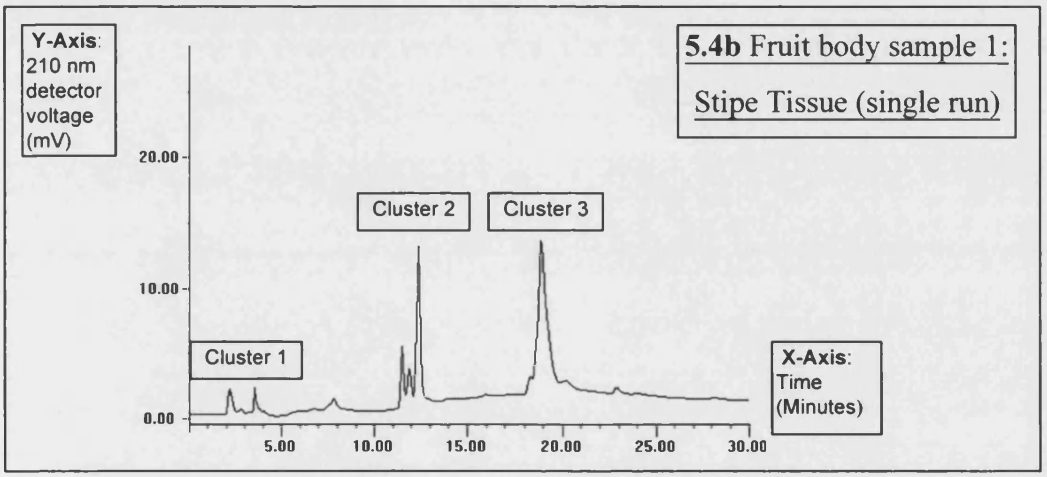




Peak identities (Ha-Hg) allocated according to Griffith *et al* 1994*b*.

Figure 5.4 Representative samples of HPLC traces for *H.fasciculare* fruit-bodies





The metabolite peaks have been grouped into clusters designated 1,2 and 3 and the peak areas in each cluster pooled as a single unit for quantitative analysis. No fruit-body metabolite peaks can be identified with peaks Ha-Hc with certainty, although Cluster 2 has a similar retention time to peak Ha and Cluster 3 a similar retention time to Hd.

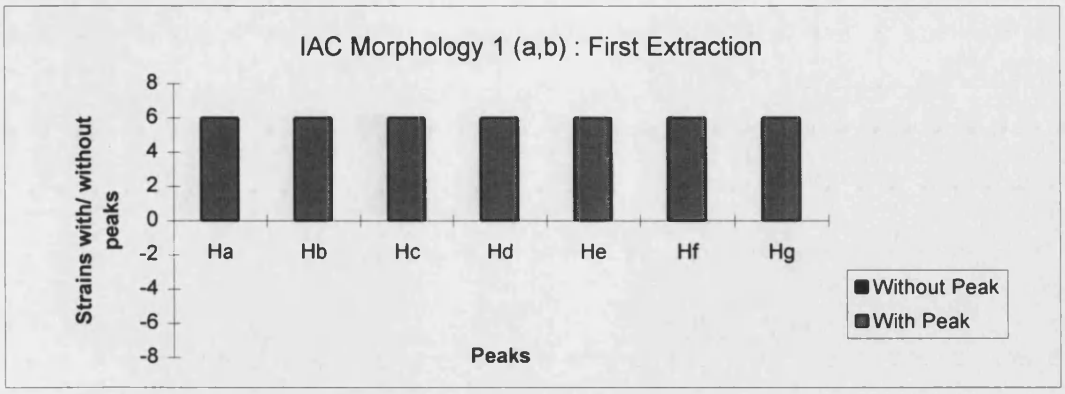
qualitative differences in metabolite peak distribution between the Class 1 and Class 2 dikaryons, between dikaryon and homokaryon classes, and between young and old cultures in all of these categories. The only peak that was constitutively present in all strains was peak Hc.

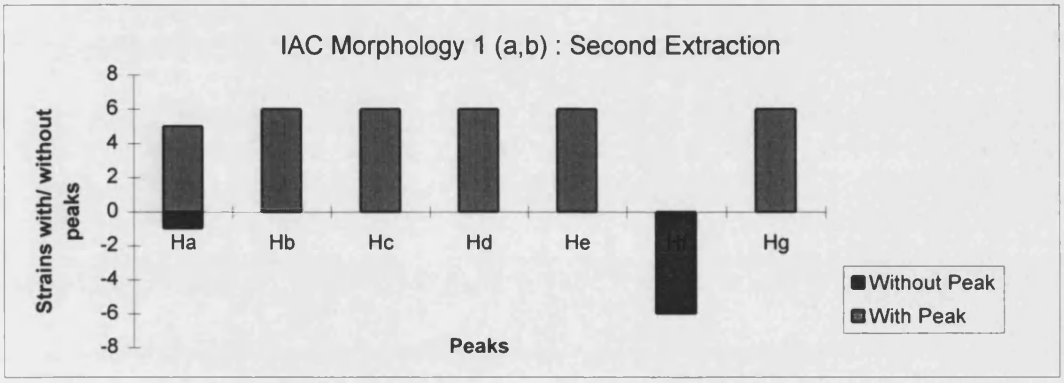
The differences in metabolite peak distribution between dikaryon cultures match with the division between Class 1 and Class 2 dikaryon morphology as summarised in ***Table 3.3***. The peak presence/ absence data in **Figures 5.5, 5.6** and **5.7** showed the reduced occurrence of many peaks in Class 2 dikaryons compared to Class 1 dikaryons. This disparity was more pronounced in the second extraction, where many Class 2 dikaryon cultures lost peaks that had been present in the first extraction. The only peak always lost by Class 1 dikaryons was peak Hf, which was present in the majority of dikaryon cultures in the first extraction but was not seen at all in the second extraction of the same cultures.

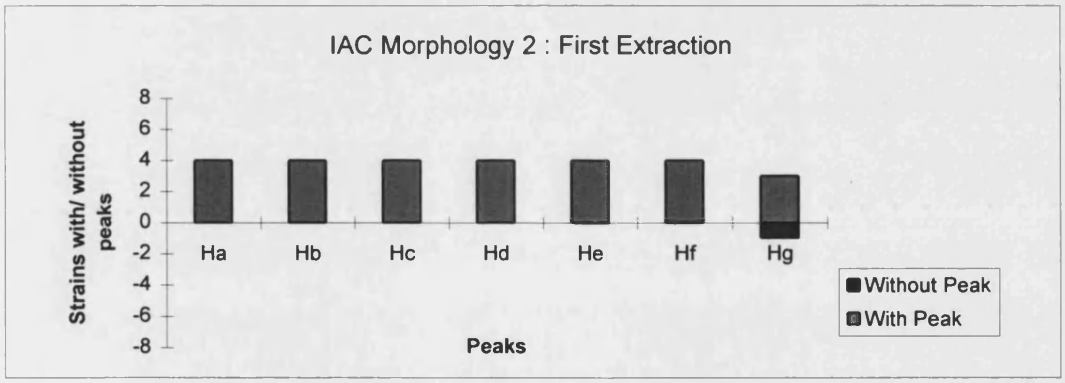
All the homokaryons tested showed a reduced number of peaks compared to both Class One and Class Two dikaryons. Very few homokaryons showed the hydrophobic peak Hg, and most did not show peaks He or Hf. These peaks, when they did occur, appeared in the first extraction and were much less prevalent in the second, following the pattern established for the Class 2 dikaryons.

HPLC of four independently collected fruit-body samples showed a conserved pattern of metabolites consisting of three clusters of peaks (**Figure 5.4**). All three clusters were present in six of eight fruit-body tissue samples analysed. The remaining two traces were flat-line, possibly due to experimental error. The three-cluster pattern was present in HPLC traces of metabolites from both cap and stipe tissue.

Figure 5.5 Peak Presence/ Absence Charts for IAC dikaryons







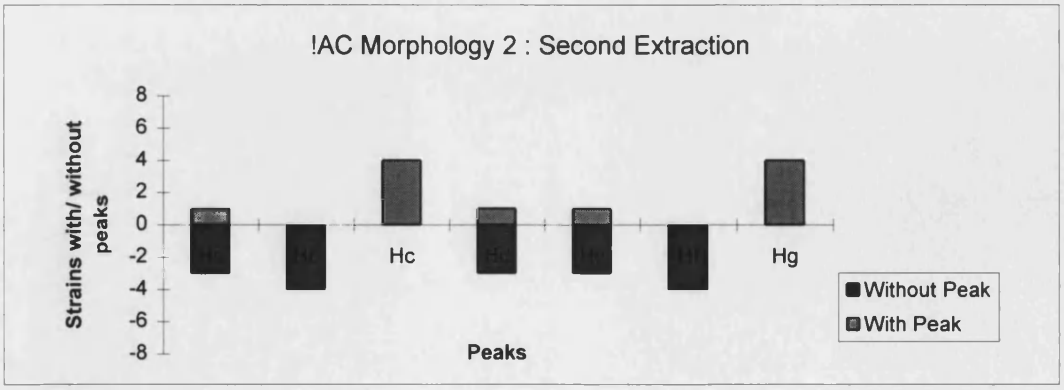
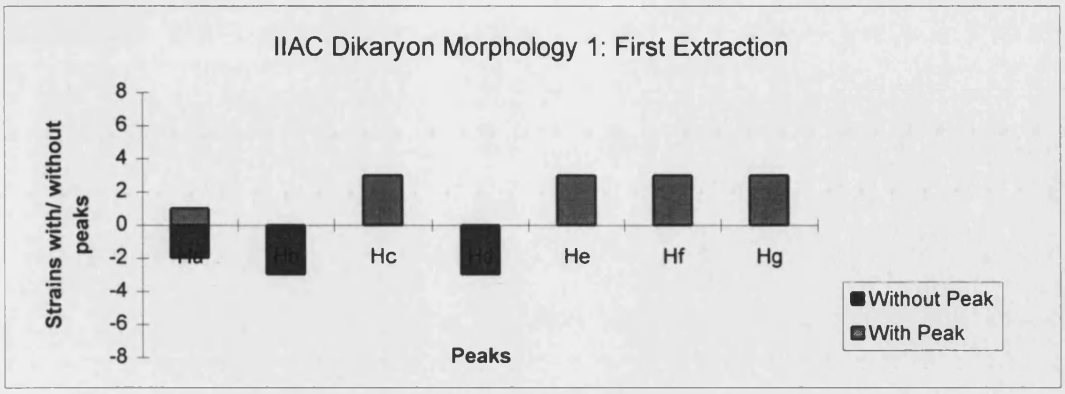
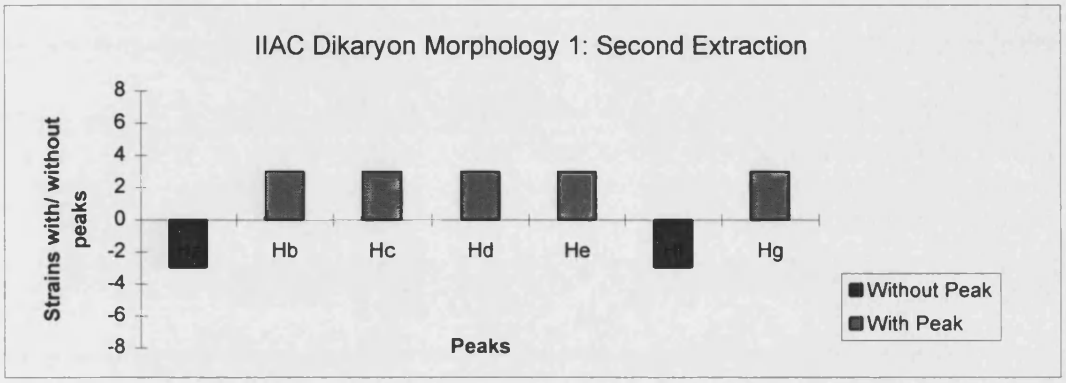
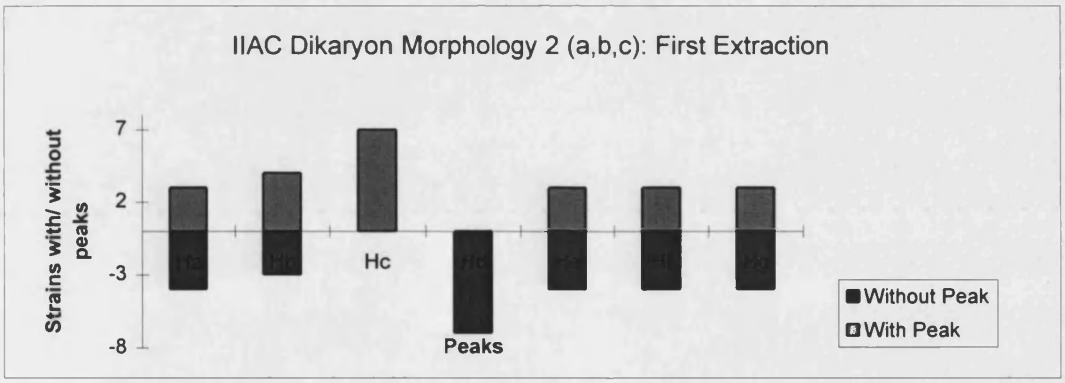


Figure 5.6 Peak Presence/ Absence Charts for IIAC dikaryons







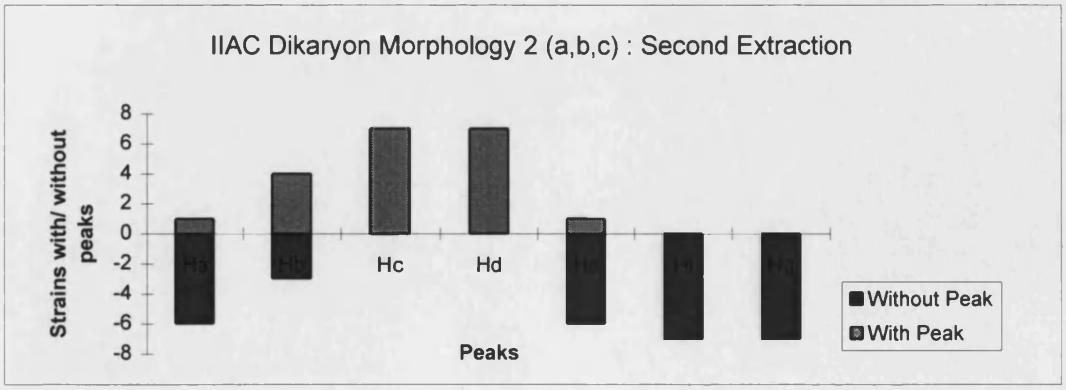
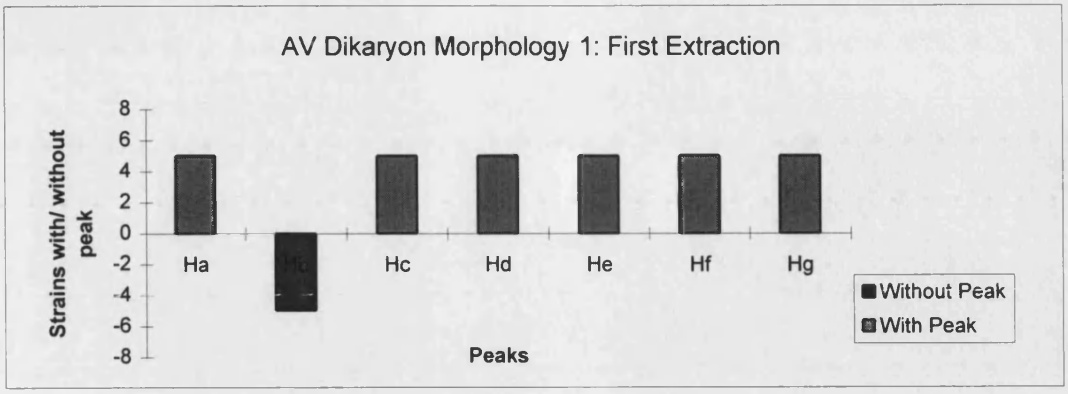
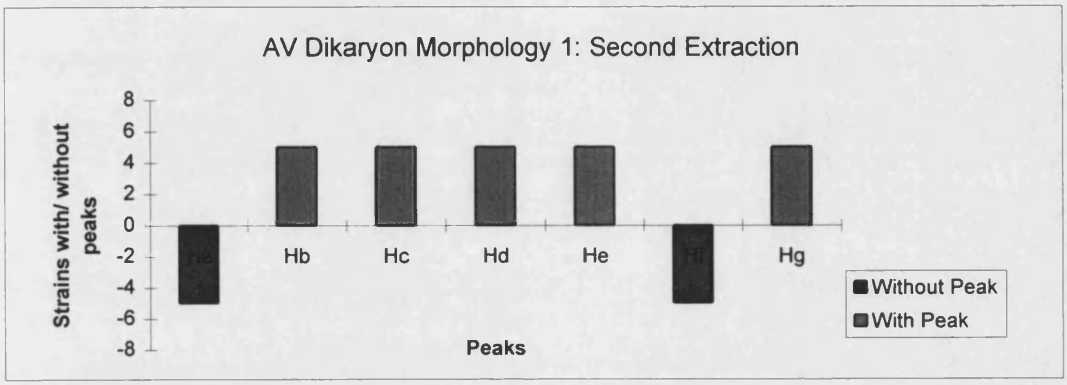
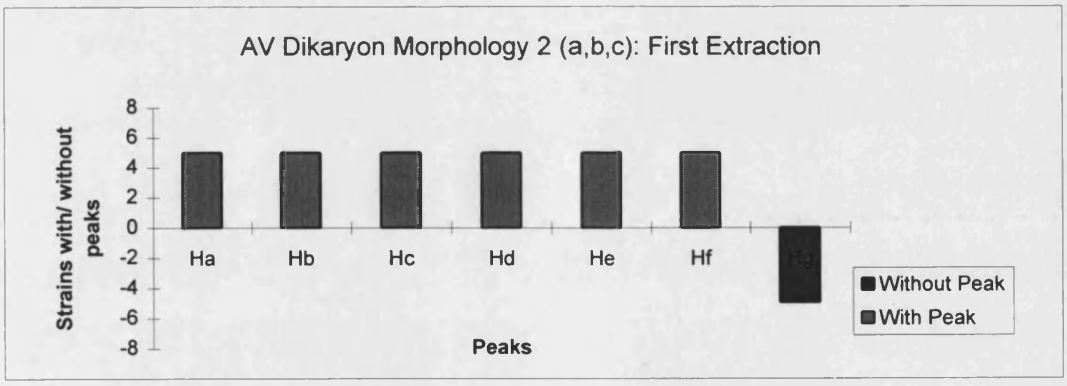


Figure 5.7 Peak Presence/ Absence Charts for AV dikaryons







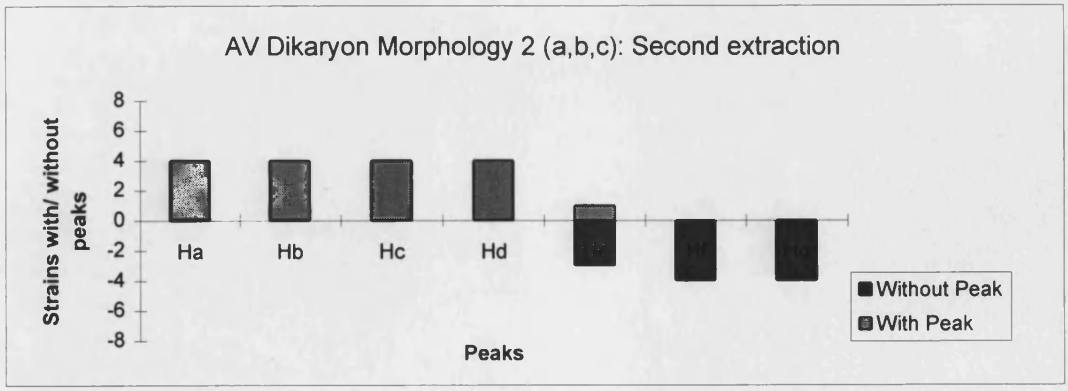
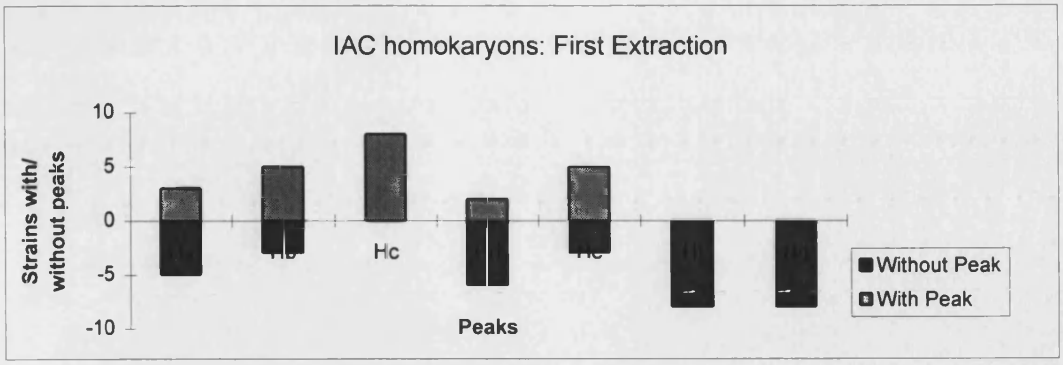


Figure 5.8 Peak Presence/ Absence Charts for IAC homokaryons



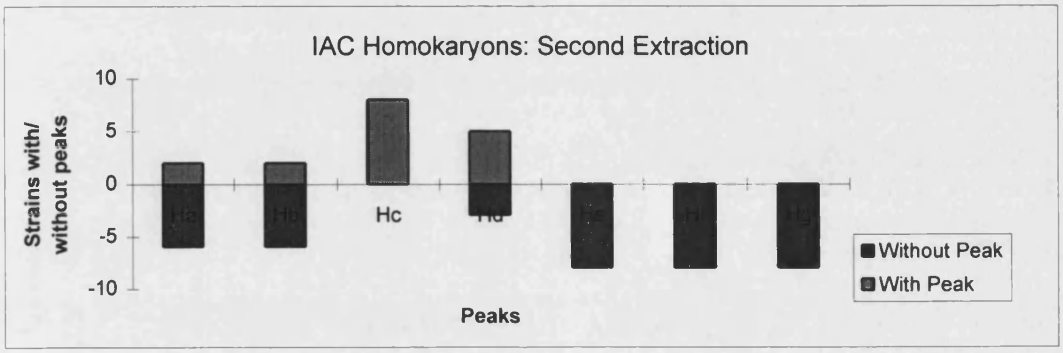
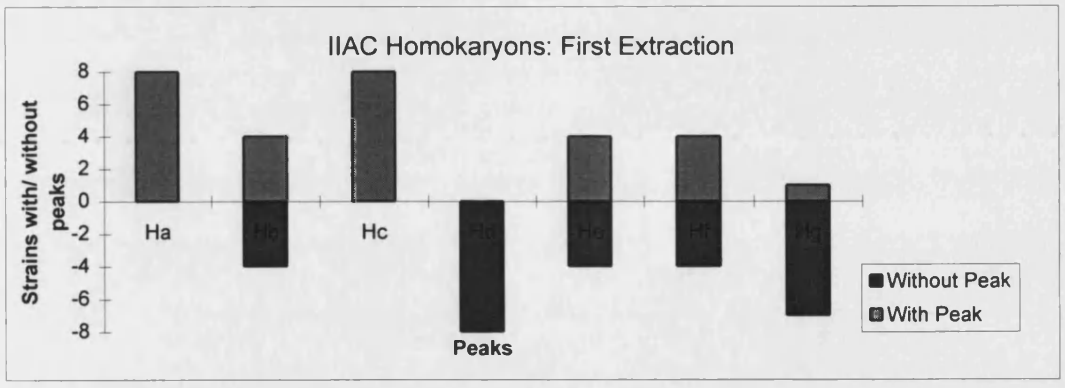


Figure 5.9 Peak Presence/ Absence Charts for IIAC homokaryons



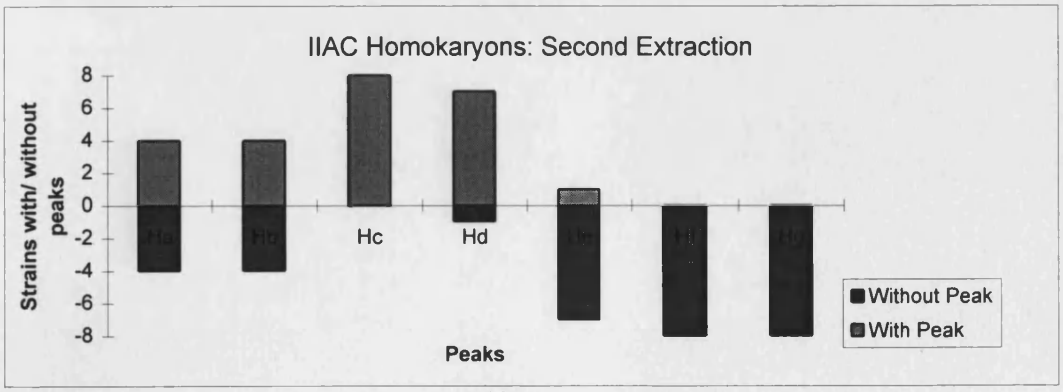
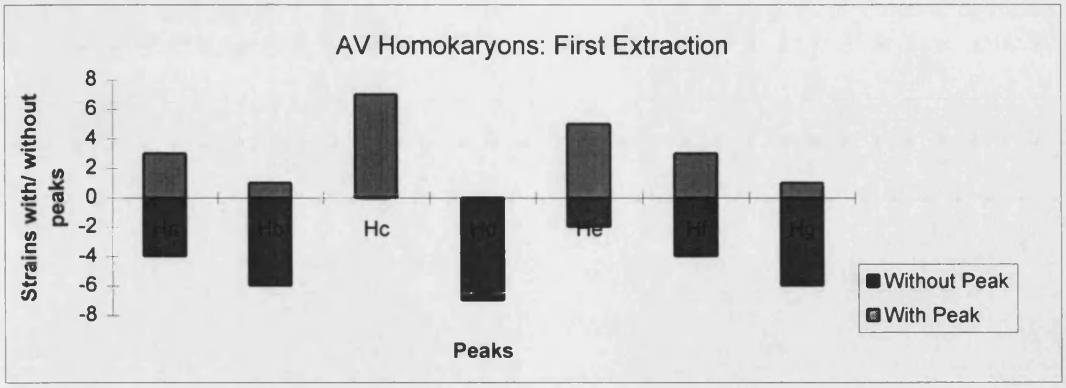


Figure 5.10 Peak Presence/ Absence Charts for AV homokaryons



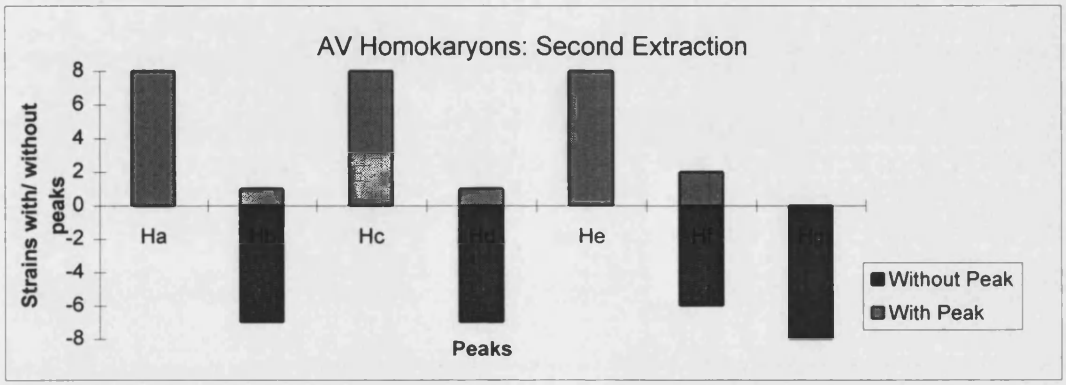


Table 5.6 Summary of peak presence/absence data (**Figs 5.5-5.10**)

Peak Identity	Percentage of cultures expressing peak in either first or second extraction.			Notes on peak prevalence
	Class 1 Dikaryons (13 Strains)	**Class 2 Dikaryons** (16 strains)	**Homokaryons** (24 Strains)	
Ha	86	75	61	Prevalence decreases with age
Hb	100	81	43	Often present but erratic
Hc	100	100	100	Always present in all cultures.
Hd	100	80	54	Prevalence increases with age
He	100	75	61	Stable in all Class One dikaryons, but prevalence decreases with age in other classes.
Hf	100	75	30	Prevalence decreases sharply with age.
Hg	100	38	9	Stable in all Class One dikaryons, but prevalence decreases with age in other classes.

The conclusion drawn from the qualitative data was that peak Hg, and to a lesser extent He, were the most strongly correlated with phenotype and merited further quantitative analysis. As peak Hc was constitutively present and abundant in all the strains tested it was selected as a reference peak against which peaks He and Hg could be compared.

Quantitative analysis of peaks Hc, He and Hg

The relative abundance of peak Hc was compared between the different time samples of Class 1 and Class 2 dikaryons (**Figures 5.11, 12, 13**). All dikaryon cultures showed a similar (+/- 27%) abundance of Hc in the first extraction. In the second extraction AV Class 1 dikaryons showed a rise in Hc of 265%, compared to the values of

between 17% and 94% recorded for the other five groups. The large Hc peak in the second AV extraction might be explained by differences in the culture incubation time; the AVS second extraction was carried out earlier (by 15 days) than the extractions for the IAC and IIAC dikaryons. This is supported by the data in Griffith *et al* 1994b, where the Hc peak was strongest in 19-day cultures and lower in 24-day cultures, possibly indicating a decrease in Hc upon longer incubation times. This does not explain why there is no large Hc peak in the Class 2 AV dikaryons, but these cultures are different in many respects and may not share the Class 1 growth or metabolite production dynamics.

The pattern of Hc production in homokaryons was similar to that of dikaryons, with IAC and IIAC homokaryon cultures (first and second extractions) giving mean Hc peaks 85-99% the size of their corresponding Class 1 dikaryon groups (**Fig 5.14**). The AV homokaryons produced a similar amount of Hc to all the other homokaryons tested, but due to the large Hc peak size of the AV Class 1 dikaryons this gave a relative abundance of only 44%. The increase in Hc between the first and second extractions was 27% for both IAC and IIAC homokaryons and 63% for the AV homokaryons. The combined quantitative data for peak Hc show that its production dynamics do not vary significantly between Class 1 dikaryons, Class 2 dikaryons and homokaryons.

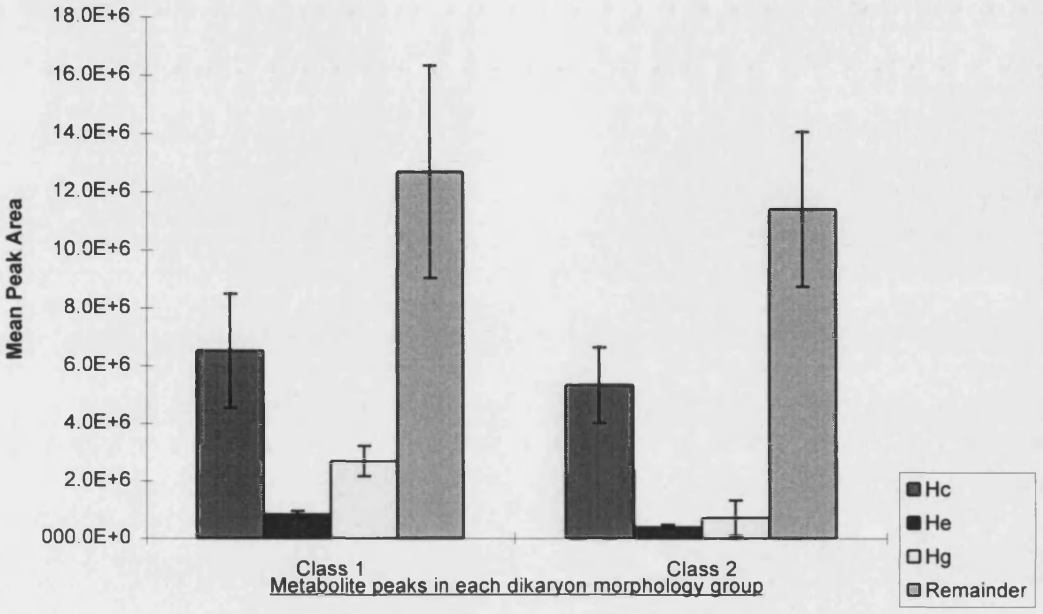
In the presence/absence tables peak He was shown to be associated with Class 1 dikaryons. The quantitative analysis supports this; in the first extraction the mean abundance of He in homokaryons and Class 2 dikaryons was 30% and 37% (respectively) of the amount in Class 1 dikaryons. In the second extraction the He production by homokaryons fell considerably in the IAC and IIAC sets, to zero and

32% of the first extraction values respectively (**Figure 5.14**). The AV homokaryon He production also fell, but only to 81% of its first extraction value. In contrast to this, the IAC and AV Class 1 and Class 2 dikaryons increased their He production by a mean of 40% between the first and second extractions (**Figures 5.11, 5.13**). The IIAC Class 1 and 2 dikaryons showed small reductions in He production between the first and second extractions; a 1% reduction in He for IIAC Class 1 dikaryons and a 13% drop for IIAC Class 2 dikaryons (**Figure 5.12**). This data shows that both the abundance and dynamics of He peaks are correlated with the morphological class of the *H.fasciculare* isolates.

The pattern of Hg production was similar to that of He, but with a larger disparity between Class 1 dikaryons, Class 2 dikaryons and homokaryons. All Class 1 dikaryons produced Hg, and all showed a very large increase in Hg production between the first and second extractions. This increase was 224% for the IAC Class 1 set, 180% for the IIAC set and 352% for the AV set. There was a large variation in Hg production amongst the Class 2 dikaryons from the three different genetic sources; the AV class 2 dikaryons showed no Hg production in either extraction (**Figure 5.13**), whilst the IIAC Class 2 dikaryons showed some Hg production in the first extraction but little in the second; 37% falling to 2.7% of the Hg produced by IIAC Class 1 dikaryons (**Figure 5.12**). The IAC Class 2 dikaryons showed a sharp increase in Hg production (535%) between extractions one and two, but still produced less Hg (63%) relative to their Class 1 counterparts (**Figure 5.11**). The production of Hg by homokaryons was extremely limited in the first extraction (averaging 3% of that of the Class 1 dikaryons) and was totally absent in the second extraction (**Figure 5.14**).

Figure 5.11: Quantitative analysis of metabolite peaks in IAC dikaryons

5.11a Mean peak areas (with 95% confidence limits) of peaks Hc, He, Hg and remainder in IAC dikaryons: First extraction (14 days)



5.11b Mean peak areas (with 95% confidence limits) for peaks Hc, He, Hg and remainder in IAC dikaryons: Second extraction (68 days)

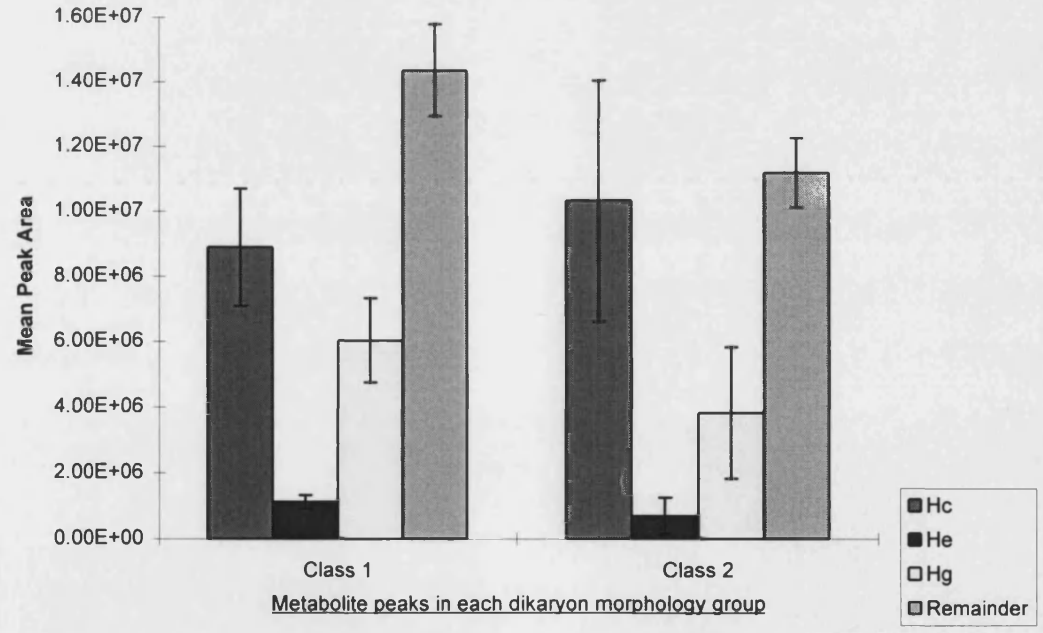
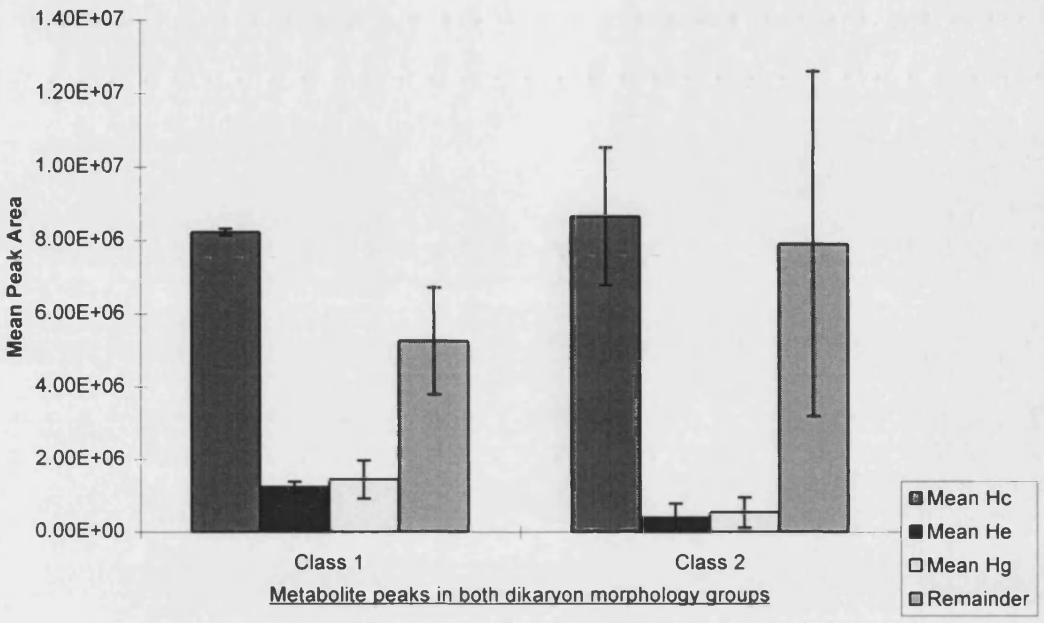


Figure 5.12: Quantitative analysis of metabolite peaks in IIAC dikaryons

5.12a Mean peak areas (with 95% confidence limits) for peaks Hc, He, Hg and remainder in IIAC dikaryons: First extraction (20 days)



5.12b Mean peak areas (with 95% confidence limits) for peaks Hc, He, Hg and remainder in IIAC dikaryons: Second extraction (65 days)

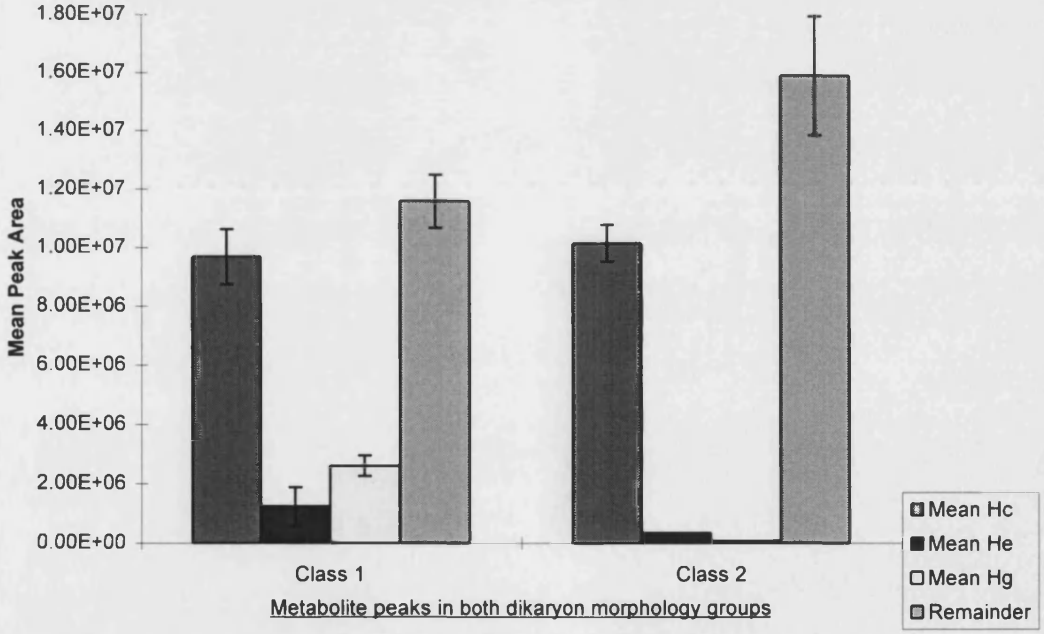
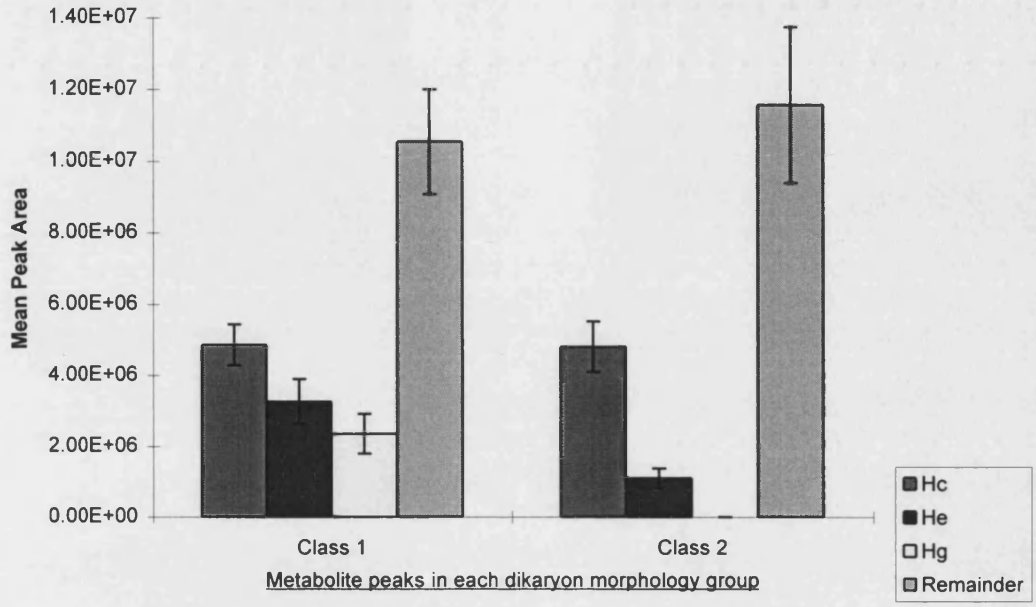


Figure 5.13: Quantitative analysis of metabolite peaks in AVS dikaryons

5.13a Mean peak areas (with 95% confidence limits) of peaks Hc, He, Hg and remainder in AVS dikaryons: First extraction (17 days)



5.13b Mean peak areas (with 95% confidence limits) for peaks Hc, He, Hg and remainder in AVS dikaryons : Second extraction (50 days)

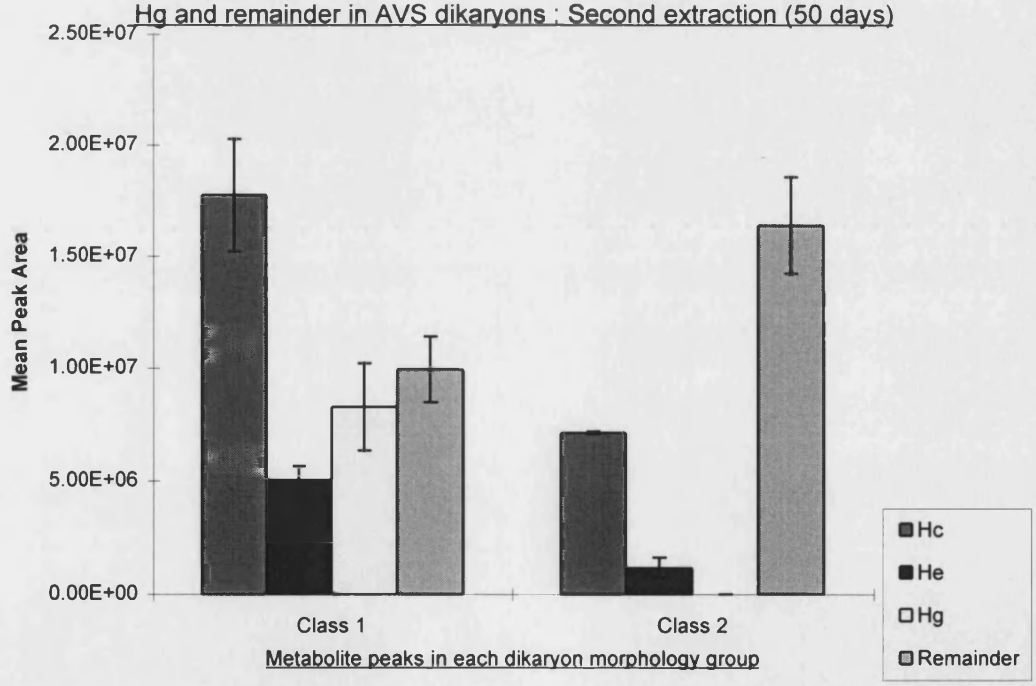
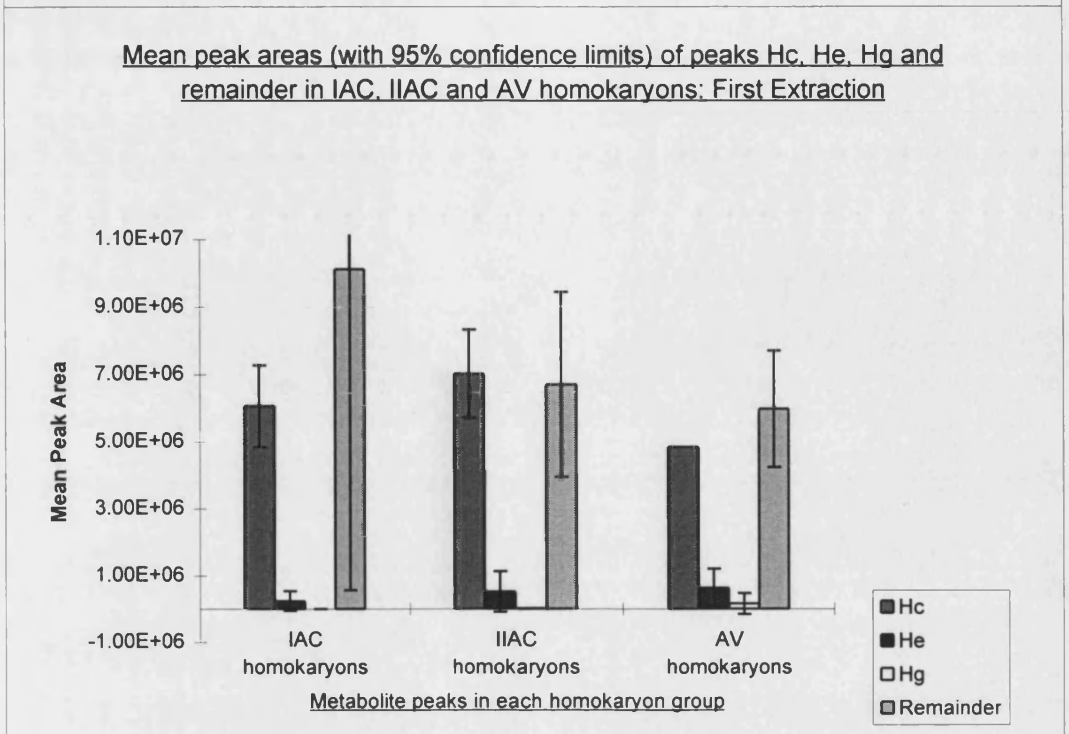
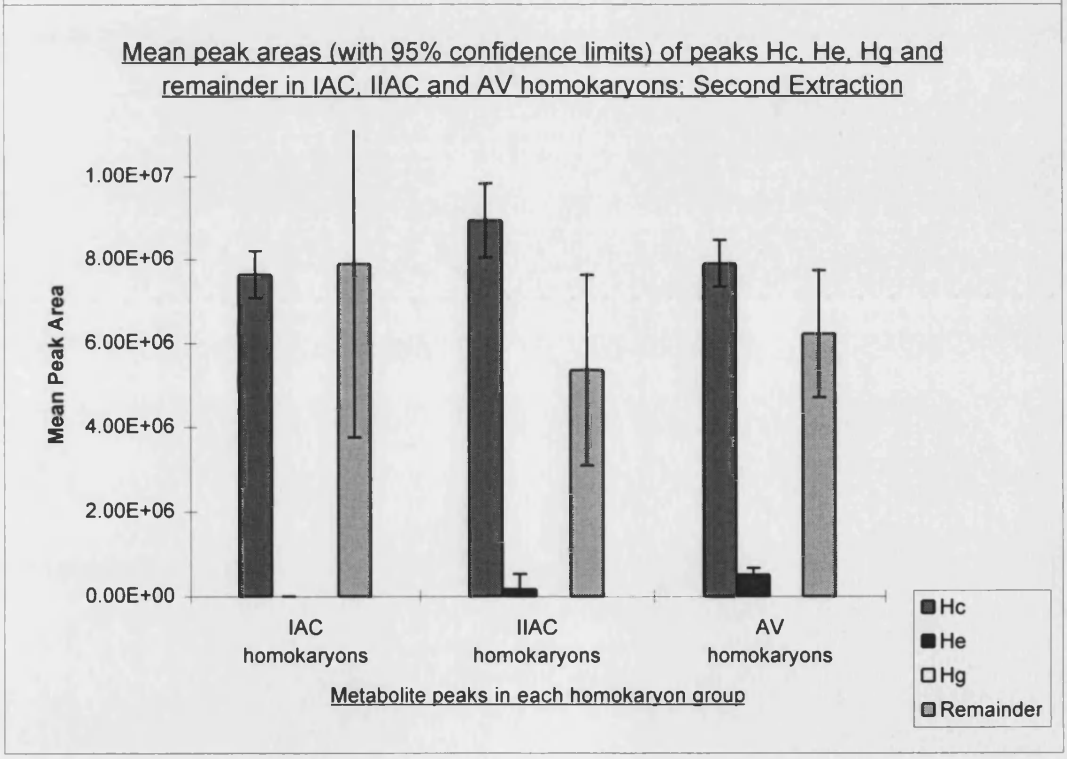


Figure 5.14 Quantitative analysis of metabolite peaks in homokaryons





Analysis of fruit-body metabolites

Representative HPLC traces of metabolites extracted from *H.fasciculare* fruit-body stipe and cap tissue are presented in **Figure 5.4**. The peaks present in these traces were not found to correspond clearly to those of either *H.fasciculare* homokaryons or dikaryons. Peak Clusters 2 and 3 show similar retention times to peaks Ha and Hd respectively, but the detailed peak structures are sufficiently different to cast doubt on any identification made between these peaks.

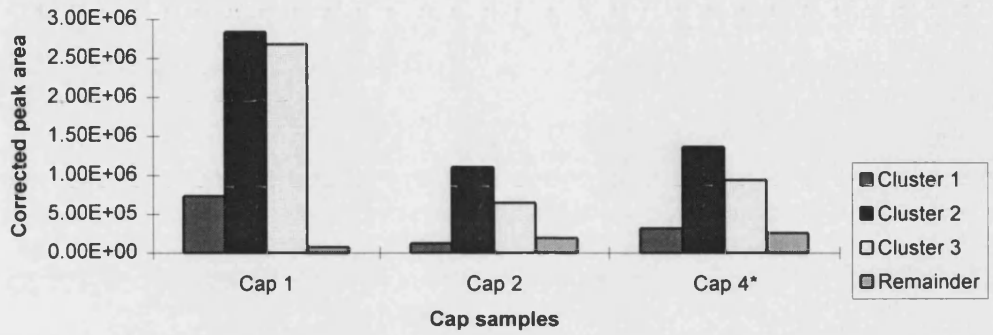
The function and development of fruit-bodies is very different from that of vegetative mycelium and it is therefore not surprising that the metabolites within them are different from those produced by the mycelium. This difference does provide some negative data in that it shows that none of the metabolites identified have basic 'housekeeping' functions such as cell-membrane components or primary metabolites.

An analysis of the metabolite peak areas for the metabolites extracted from *H.fasciculare* fruit-body tissue is presented in **Figure 5.15**. The total metabolite peak area for each of the six samples was approximately proportional to the wet weight of the fruit-body component, as shown in **Figure 5.15c**. The peak data were adjusted to account for variations in the wet-weight of the samples used for HPLC extraction. The first conclusion that can be drawn from these data is that the peak clusters 1, 2 and 3 are present in approximately similar proportions in fruit-body samples from different sites, with each site presumed to represent a different *H.fasciculare* genetic individual.

The second conclusion that can be drawn is that metabolite distribution in *H.fasciculare* fruit-bodies shows a correlation between the tissue differentiation state and the distribution pattern of metabolites. Fruit-body cap tissue showed a reduced

Figure 5.15: Metabolite peak area (corrected for wet weight) in *H.fasciculare* fruit-body components.

5.15a Metabolite peak area (corrected for wet weight of) for H.fasciculare fruit-body cap metabolite extractions



5.15b Metabolite peak area (corrected for wet weight) for *H.fasciculare* fruit-body stipe metabolite extractions

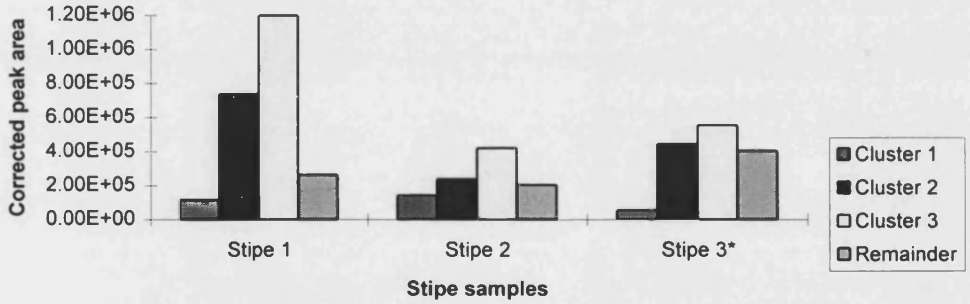
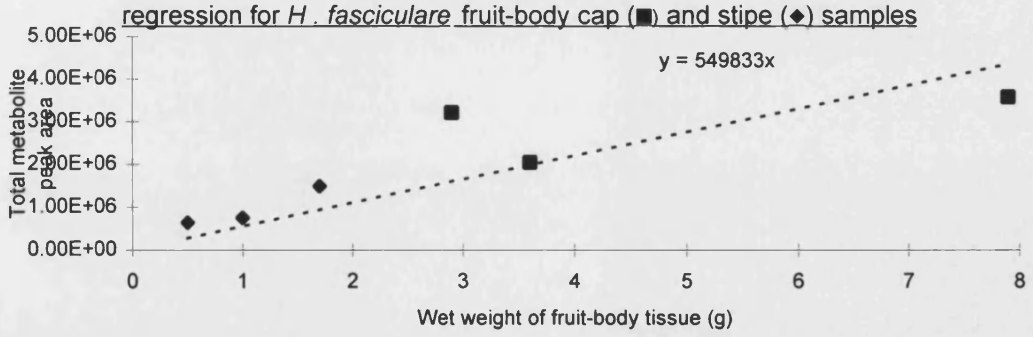


Figure 5.15c Total metabolite peak area against wet-weight with linear regression for *H . fasciculare* fruit-body cap (■) and stipe (◆) samples



Corrected metabolite peak areas were calculated in each case by multipying the raw value by (total metabolite area / (wet weight x m), where m is the value derived in **Fig.5.15c** by linear regression.

* No data was available for samples Cap 3 and Stipe 4
(flat-line HPLC trace possibly due to experimental error)

proportion of the Cluster 3 metabolites (hydrophobic) compared to the stipe tissue. Stipe tissue has a function of structural support and contains 'conduit' hyphae that allow for the rapid throughput of resources; such a tissue might be expected to be richer in insulative, strengthening cell-wall components. The increased Cluster 3 metabolites may represent, or be associated with, such components.

General Conclusions from *H.fasciculare* HPLC data

The results of this HPLC study of metabolite distribution indicate that *H.fasciculare* phenotype is correlated with the production and distribution of hydrophobic metabolites, an observation that is consistent with the 'oxidative stress' model of mycelial morphogenesis presented in the General Introduction. The metabolite/phenotype correlation can be observed in *H.fasciculare* strains from disparate genetic sources and is therefore unlikely to be an artefact of irregular, chance patterns of metabolite production.

The quantitative data for peaks Hc, He and Hg confirm that the production of two metabolites (He and Hg) is associated with phenotypic class in *H.fasciculare*, and that the production of another metabolite (Hc) is relatively independent of phenotypic class. Peaks He and Hg are both relatively hydrophobic, as revealed by their retention time within the HPLC solvent gradient, and are thus candidates for boundary-insulating compounds. The phenotype that He and Hg are most strongly associated with is the Class 1 (normal) dikaryon, a mycelial organisation with the ability to generate rapidly extending aerial hyphae, mycelial cords and fruit-bodies. Most of these functions are lacking in the Class 2 dikaryons and homokaryons, and these phenotypic classes have either no He/Hg or a reduced He/ Hg production that often decreases over time.

Although the difference between the biochemistry of the dikaryotic and homokaryotic life-cycle stages of basidiomycetes is rarely emphasised in the literature, this HPLC data shows that the obvious morphological differences between the two *H.fasciculare* life-cycle stages are mirrored by significant biochemical differences.

There is an interesting correlation between genetic stability and the presence of peak Hg. In Chapter Four it was shown that no somatic recombination occurred within IAC conidial homokaryons, while in this chapter it was seen that the IAC Class 2 strains showed a higher retention of peak Hg than the Class 2 strains of the IIAC and AV strains. This could be interpreted in two ways; the first is that Class 2 strains lacking Hg are generated by somatic recombination. The second interpretation is that strains better able to stably express the insulative Hg metabolites are protected against stress-induced genomic damage. This latter theory raises the possibility that secondary metabolite production may be correlated with genomic stability, a counterintuitive proposition within the conventional framework of genetics but an entirely reasonable one within the oxidative stress/ boundary chemistry model. An experiment to resolve these possibilities might be instructive.

The data presented here provide strong circumstantial evidence for the function of hydrophobic metabolites in the *H.fasciculare* mycelium. However, more direct evidence needs to be gathered if this hypothesis is to be proved, and that will demand detailed information on the chemical structure, localisation and (epi)genetic control of these metabolites.

Chapter Six

HPLC profiles of *H.fasciculare* strains: Phenotypic and Functional Relationships

Introduction

The previous chapter outlined the main metabolite-profile differences between the homokaryon, dikaryon and fruit-body life-cycle stages of *H.fasciculare*. In addition, a distinction was made between Class 1 and Class 2 dikaryons derived from regenerated fruit-body tissue. The metabolite peak that was found to correlate best with mycelial morphology in *H.fasciculare* was peak Hg. The position of this peak on the HPLC chromatograms indicated that it was relatively hydrophobic, but apart from this limited information nothing was known about it. The experiments detailed in this chapter are aimed at clarifying the role of the Hg metabolite peak.

The first results section in this chapter is a comparison of Hg peak structures in different strains of *H.fasciculare* as revealed by HPLC chromatograms. This was carried out to determine if differences in the size and shape of the Hg peak could be correlated with dikaryon morphology within and between the various classes of dikaryon already identified. Variations in the occurrence of the strong, 'erratic' peak Hb are also considered in this section. The second results section in this chapter addresses the issue of metabolite localisation. Clearly, if an 'insulative' role is predicted for a metabolite then it should show some degree of association with the hyphal wall. To establish whether or not this was the case, a liquid culture regime was combined with HPLC analysis in order to distinguish between metabolites released into the growth medium and those sequestered by the mycelium. The third section is an attempt to correlate the inter-class metabolite differences already discussed with a biologically significant process, that of interspecific combat between *H.fasciculare* and other fungal species. Previous sections have discussed the hypothesis that insulative compounds are

an important factor in determining K-selected (combative/defensive) status in fungal mycelia; if the metabolite profiles of *H.fasciculare* strains can be correlated with combative ability then this would provide further circumstantial evidence for the importance of these compounds in the generation of phenotype.

Methods

Presentation of HPLC data

The chromatograms from the HPLC analysis carried out in Chapter Five were examined for the correlation of particular Hg peak structures with morphological classes of *H.fasciculare* dikaryon. Representative HPLC chromatograms were selected, enlarged and are illustrated in **Figures 6.1** and **6.2**. Chromatograms showing large Hb peaks were also selected and are shown in **Figures 6.3** and **6.4.**

HPLC analysis of liquid-grown dikaryons

A detailed procedure for this is described in General Methods. In summary, isolates of *H.fasciculare* were grown in still liquid culture. The mycelial and liquid phases of the culture were separated by filtration, metabolites were then extracted from each fraction and analysed by HPLC. The location of the metabolites was established by comparing the proportions of metabolites found in the profiles from the liquid and mycelial phases of each culture.

Pairing experiments

H.fasciculare strains (see ***Table 6.1***) were paired against a heterokaryotic isolate of *Phlebia radiata* and fungus KB, an aggressive strain of unknown species initially isolated as a laboratory contaminant. The fungi were paired approximately 4cm apart in the centre of 9cm 2MA plates. One pairing plate was made for each strain combination. Since KB and *P.radiata* grew more rapidly than *H.fasciculare*, the latter was allowed to establish for 7 days prior to the inoculation of either antagonist. The isolation of strains, subculturing procedure and incubation conditions are described in General Methods. Photographs were taken of some developing interactions after 27 days, and the final outcome of the pairing interactions was photographed after 76 days; by this point no visible change had occurred in the colonies for a week.

Table 6.1 : H.fasciculare strains used in pairing experiments

Source	Class 1 dikaryons	Class 2 dikaryons	Basid. isolate homokaryons
IAC	1a: S1A S1C 1b: S1F S1L S1H S3C* S3D	S1D S2D	34, 40 44, 47
IIAC	S4C, S6C, S6D, S7B	S7C, S7M, S7D1, S7H	84, 124, 136 140
AV	1E 1F 1G 3D	1K2, 3L*, 2B, 2K	1, 7, 9, 10

*Due to an error, these strains were paired against *P. radiata* only.

Results and Discussion

Variations in the Hg peak complex

The detailed structure of the Hg peaks of several strains is shown in **Figures 6.1** and **6.2**. The largest peaks are exhibited by IAC Class 1a and AV Class 1 dikaryons. Both of these have a relatively symmetrical 3-peak structure, with a large central peak and two subsidiary peaks, one on each side. The retention time of the highest segment of these peaks is typically 26.5-27 minutes. The IIAC Hg peak does not follow this pattern; it is very much smaller (approximately 33% the size of the IAC and AV peaks) and made of three comparatively widely-spaced sub-peaks, the first being much larger than the second two. Neither the IIAC or AV genetic sources gave rise to more than one morphological class of dikaryon exhibiting the Hg peak. However, three classes of IAC dikaryon were found to produce Hg. The Class 1a Hg peak has been described above, but Class 1b and some Class 2 dikaryons also produced an Hg peak. If these are compared to the 1a peak **(Figure 6.1)** it can be seen that the Class 1b and 2 Hg peaks are much smaller, less symmetrical and have a retention time retarded by about 30 seconds.

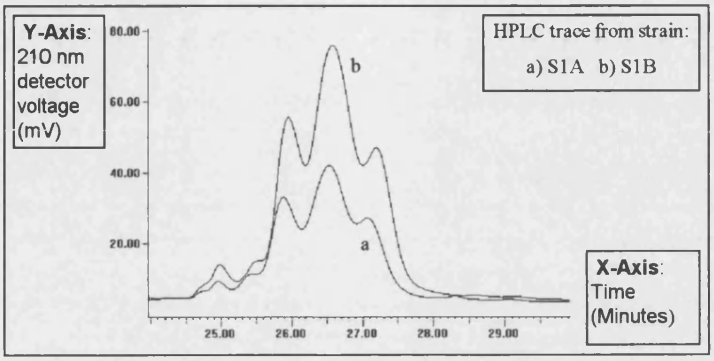
In Chapter Five the occurrence and size of metabolite peak Hg was seen to correlate with the phenotypic characteristics of Class 1 and Class 2 dikaryons. The data presented here indicates that the substructure of peak Hg may also be correlated with phenotypic class. Specifically: IAC Class 1b strains have reduced mycelial cord formation compared to IAC Class 1a strains; IAC Class 2 are 'flat' strains, and the IIAC Class 1 dikaryons come from a genetic source that seems to have dysfunctional meiotic processes. All of these phenotypes are associated with Hg peaks different from

the presumed 'normal' peak structure of IAC Class 1a and AV Class 1 dikaryons. If this data is taken as a whole, it might be suggested that the Hg peak is a barometer of dikaryotic functionality.

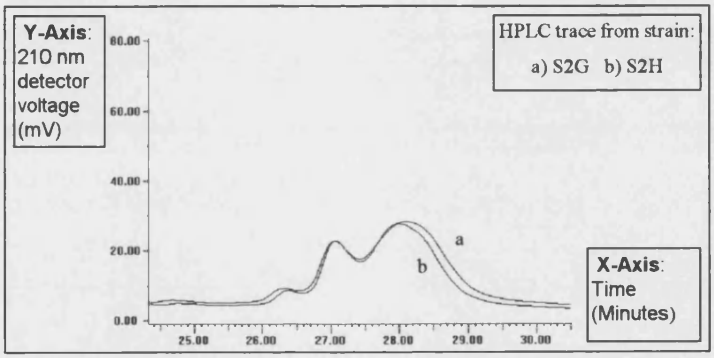
Erratic and dikaryon-class occurrence of the Hb and novel 'Hh' peak

The occurrence of the Hb peak was recorded in the presence/absence tables in Chapter Five, where it followed no easily identifiable pattern. In cases where Hb did appear, its size was also extremely variable, ranging from 'just identifiable' to the largest peak on the chromatogram. Trace-by-trace analysis of the data revealed that large Hb peaks were associated with IAC Class 1b dikaryons (**Figure 6.3**) and a subset of basidiospore-derived homokaryons from all three genetic sources. The occurrence of a large Hb peak in IAC Class 1b dikaryons provides another marker for that morphological class in addition to the altered Hg peak discussed above. It is also the only case of a peak gain, as opposed to the more usual losses, in a variant morphological class when compared to the normal 1a or 1 dikaryon classes. The occurrence of Hb in homokaryons included two dramatic examples of IAC basidiospore isolates where the Hb peak was larger than all other peaks combined; this was associated with crystal formation in ethyl-acetate metabolite extracts but no obvious morphological characteristics (**Figure 6.4-*1***). These large Hb peaks accounted for the high variability in the quantitative analysis of the IAC basidiospore homokaryon's metabolite profiles (Chapter Five, **Figure 5.14**). Another feature of these IAC basidiospore homokaryon profiles was the presence of a novel peak (termed 'Hh') with a retention time of 16.5 minutes, partially merged with peak Hc. This peak was not seen in

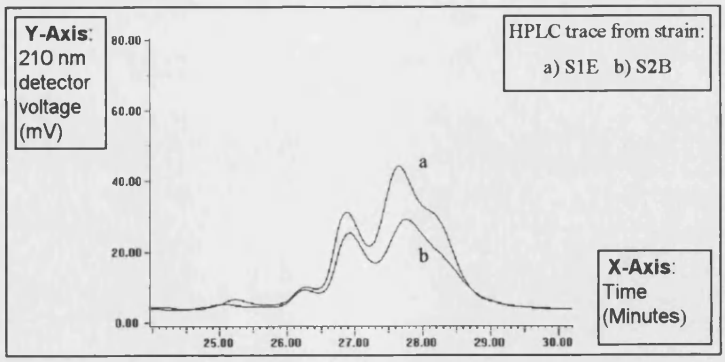
Figure 6.1 : Comparison of Hg peak structure in IAC class 1a, 1b and 2 dikaryons



1) Hg peaks in IAC Class 1a dikaryons (second extraction)

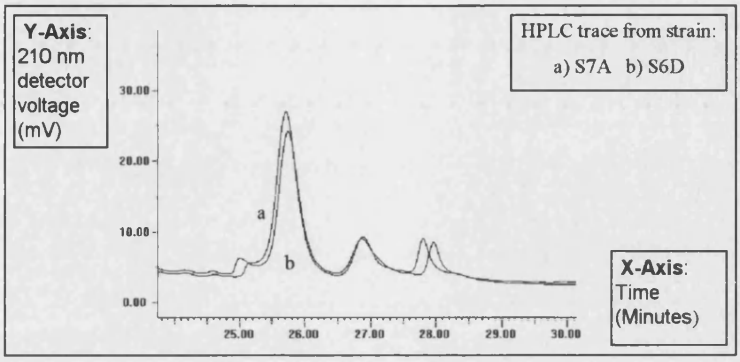


2) Hg peaks in IAC Class 1b dikaryons (second extraction)

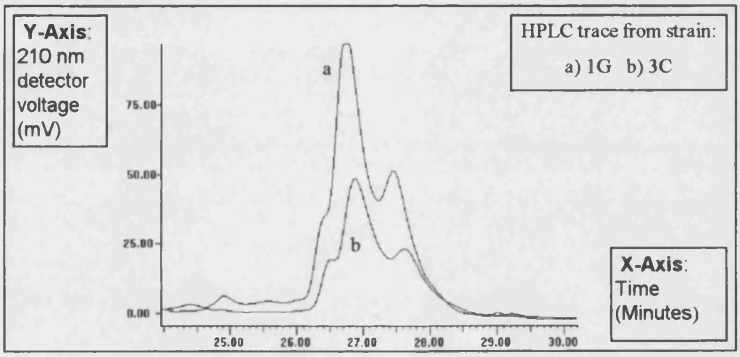


3) Hg peaks in IAC Class 2 dikaryons (second extraction)

Figure 6.2 : Comparison of Hg peak structure in IIAC and AV Class 1 dikaryons



1) Hg peaks in IIAC Class 1 dikaryons (second extraction)



2) Hg peaks in AV Class 1 dikaryons (second extraction)

any other homokaryon isolates, but was present in all the IAC dikaryon isolates (**Figure 6.3**).

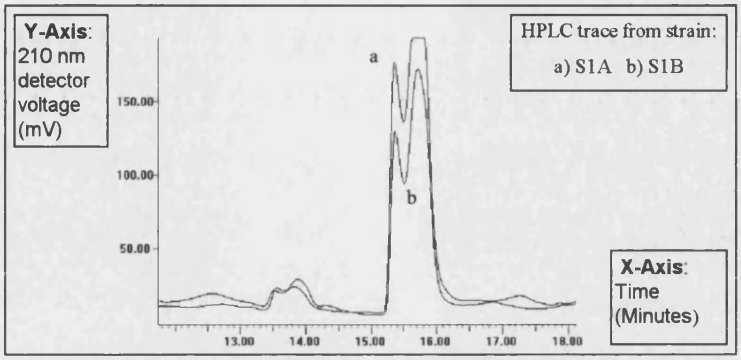
Since Hb occurs only erratically , it cannot represent an important metabolic or structural component of any of the life-cycle stages of *H.fasciculare*. In some cases its excess production is reminiscent of the massive (+)-torreyol formation in degenerating *Stereum hirsutum* strains after unstable mating interactions (Ainsworth and Rayner 1989), but in this case there is no apparent genomic instability or senescence reaction. In the case of such large, erratic peaks it is worth restating this caution: Peaks with the same retention time in an HPLC system may not represent the same metabolite. The occurrence of Hb and Hh is interesting but requires more discriminating analysis than can be performed with the techniques used here.

Results of the liquid culture experiment

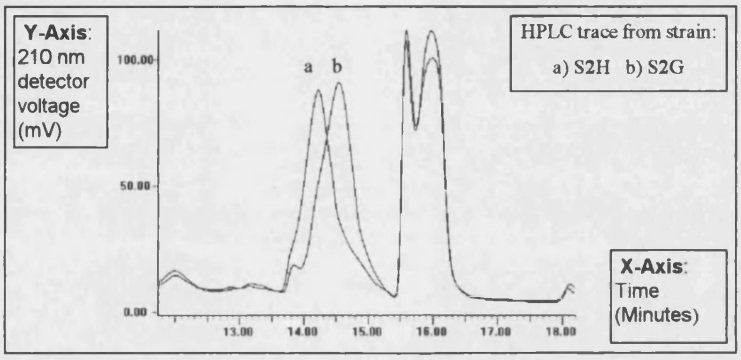
The results of the HPLC analysis of the mycelial and liquid phases of Class 1 dikaryon cultures are presented in **Figure 6.5**. Although only nine strains were used in the experiment there are clear differences in the metabolite peak distribution between genetic sources and mycelial and liquid phases.

As with solid-phase cultures, the largest metabolite peak produced was Hc. The majority (86%) of this was found in the liquid phase of the cultures; this proportion did not vary significantly between dikaryons from different genetic sources (mean range of ± 6%). The small proportion of Hc left in the mycelial phase may not be significant as it could reflect a residue of liquid phase trapped within the mycelial mass.

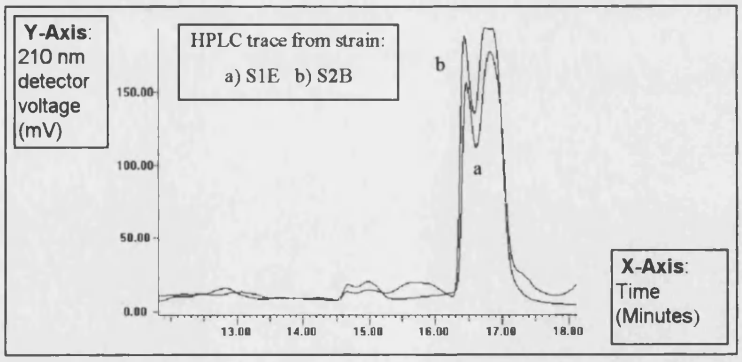
Figure 6.3 : Comparison of Hb and Hc peak structures in IAC dikaryons



1) IAC Class 1a dikaryons (second extraction)

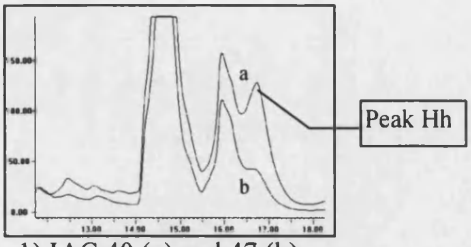


2) IAC Class 1b dikaryons (second extraction)

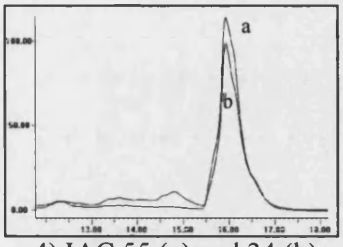


3) IAC Class 2 dikaryons (second extraction)

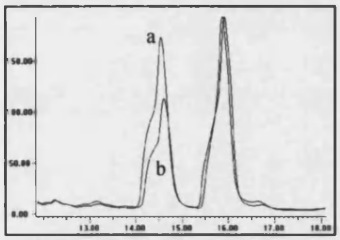
Figure 6.4 : Peak Hb occurrance in basidiospore-derived homokaryons



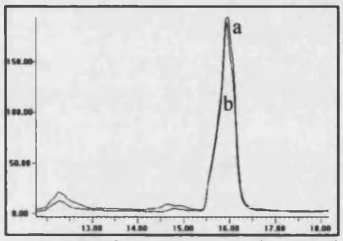
1) IAC 40 (a) and 47 (b)



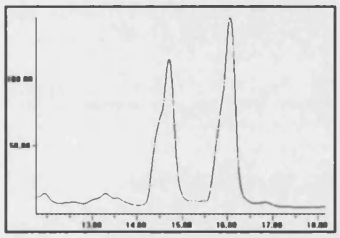
4) IAC 55 (a) and 34 (b)



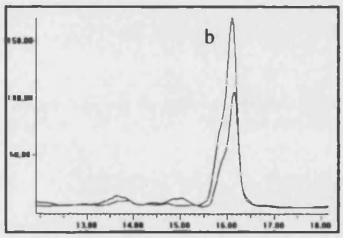
2) IIAC 75 (a) and 94 (b)



5) IIAC 149 (a) and 136 (b)



5) AV 23



6) AV 10 (a) and 12 (b)

All results are from the from first metabolite extraction (14 days).

The X and Y axes are : X=time (minutes); Y= 210nm [a] detector signal (mV)

1, 2, 3- Chromatograms of isolates showing peaks Hb (RT=14.5min) and Hc (RT=16 min). Peak Hh is also indicated in illustration 1.

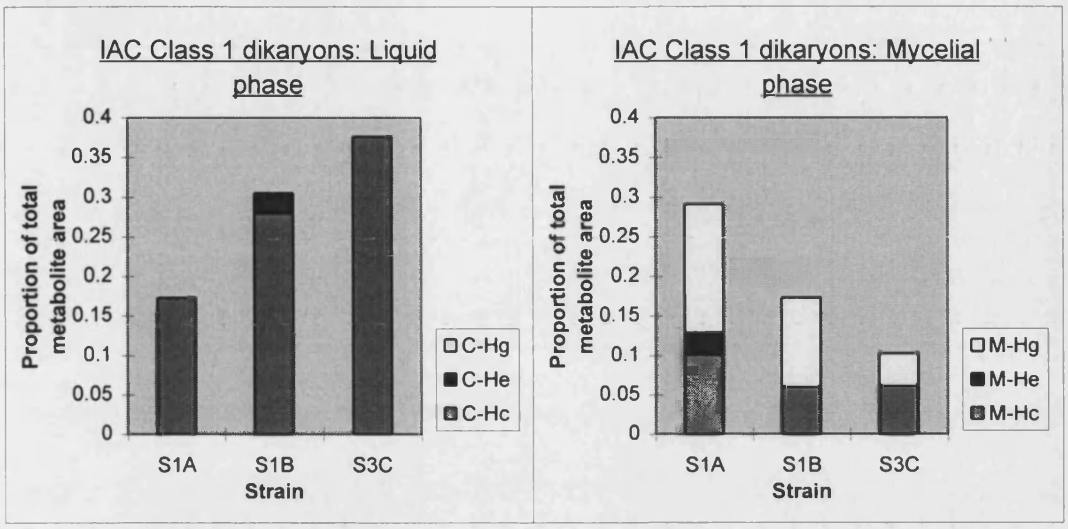
4, 5, 6-Chromatograms of isolates showing peak Hc (RT=16min) only.

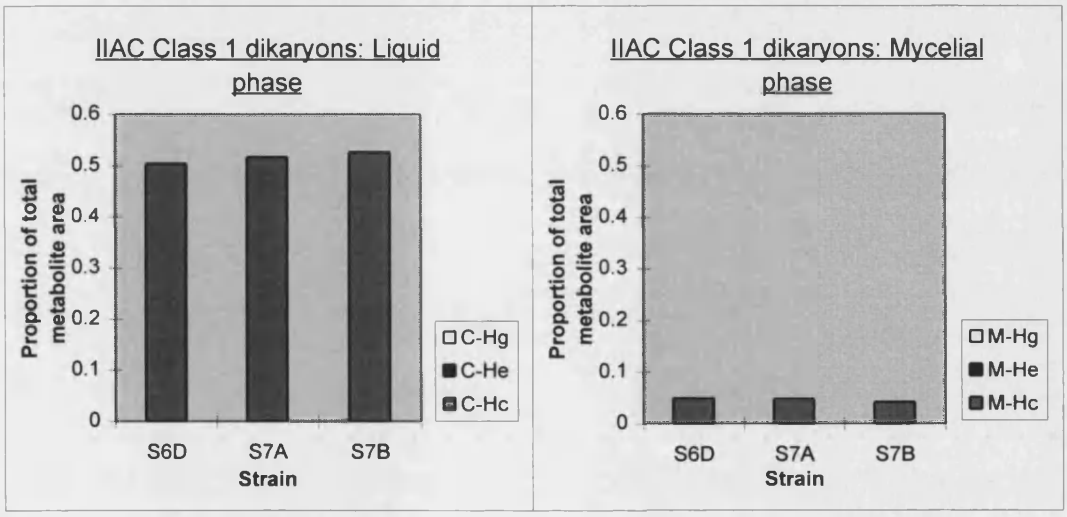
In contrast to peak Hc, peaks He and Hg were retained in the mycelial phase of the cultures, and their presence was dependent on the genetic source of the dikaryons. Their production was also different from that encountered in solid-media cultures of the same strains. Only AV dikaryons consistently produced a large proportion of peak He, and 85% of this was retained in the mycelial phase of the liquid cultures. IIAC dikaryons did not produce any He, and IAC dikaryons produced a small amount of He with an erratic distribution that may represent a mis-identified peak, possibly a shifted Hf. Peak Hg was only produced by the IAC Class 1 dikaryons, and 100% of this was retained in the mycelial phase of the cultures. Although IIAC and AV Class 1 dikaryons produced Hg in solid culture, none of them produced Hg in this liquid-culture experiment.

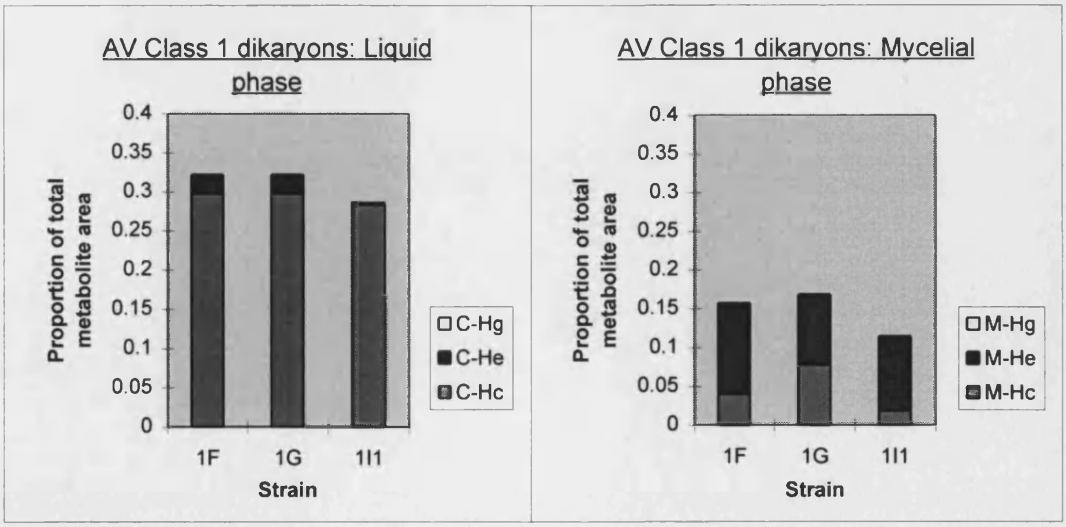
The first important conclusion that can be drawn from this experiment is that peaks Hg and He are associated with the mycelium and are not exported into the growth medium. This is precisely the location that would be expected for metabolites performing an insulative function. These results also show that He/Hg production is not constitutive in Class 1 *H.fasciculare* dikaryons, but depends on culture conditions. All the strains used produced both He and Hg when grown in solid culture. However, in liquid culture the IAC set produced only Hg, the AV set produced only He and the IIAC set produced neither. What could explain this reduction in hydrophobic metabolites? There are two obvious potential causes; the first is exposure to air and the second is growth pattern. In solid culture, a large proportion of the mycelium is aerial. This mycelium, as previously discussed, may be subject to greater oxidative stress than submerged mycelium due to the faster diffusion of oxygen through a gaseous phase

and this may stimulate the production of insulative metabolites. In contrast, submerged mycelium may be protected from oxidative stress by its aqueous environment and is therefore less likely to produce such metabolites. In terms of growth pattern, the type of inoculation used is important. In all the solid-media cultures, inoculation was by the inclusion of a single plug of colonised agar, whereas in liquid culture it was achieved by the addition of a 'paste' of colonised agar (see Chapter 6 methods). This means that the liquid cultures were comprised of many small, independent growth foci as opposed to the single, evenly extending mycelial front in solid cultures. The liquid cultures may therefore have a faster initial growth rate and a sharper transition to nutrient-limiting conditions. This may have led to a different pattern of differentiation and metabolite production within the mycelium when compared to agar cultures.

Figure 6.5 : Distribution of metabolite peaks Hc, He and Hg between liquid and mycelial phases in 21-day liquid cultures of Class 1 dikaryons







Results of interspecific pairing experiment

H.fasciculare can react to fungal antagonists by the production of invasive mycelial cords or replacement fronts. In interspecific interactions, cords extend over the surface of the competitor mycelium and allow *H.fasciculare* mycelium to penetrate into the more vulnerable internal regions, whereas replacement fronts are broad bands of slowly, often rhythmically advancing mycelium that gradually push into the antagonist's periphery. As cord production is a localised, translocative process the areas of *H.fasciculare* mycelium acting as sinks may become more susceptible to reciprocal invasion, as has been demonstrated in patterns of interaction with *Coriolus versicolor* (Griffith *et al* 1994*a*). Representative outcomes of the pairing experiments between *H.fasciculare* and both KB and *P.radiata* are shown in **Figures 6.6** and **6.7**. *H.fasciculare* isolates produced both cords and replacement fronts in these interactions and gained territory in the majority of cases. The success of each *H.fasciculare* strain in replacing the antagonist species was scored using an arbitrary point system; these results are shown in ***Table 6.2 (a,b)***; the mean scores calculated from this for each morphological class of *H.fasciculare* are shown in ***Table 6.3***. The interaction patterns that developed were dependent on the class of *H.fasciculare* isolate challenged, but relatively independent of the genetic source of the *H.fasciculare* isolates.

The highest-scoring and most combative class of *H.fasciculare* isolate were the Class 1 dikaryons, which showed equally high combative scores against both antagonists. In pairings with *P.radiata* the Class 1 dikaryons invaded *P.radiata* mycelial territory by the overgrowth of dense pale cord systems. In most cases *P.radiata* was completely replaced by *H.fasciculare* during the course of the

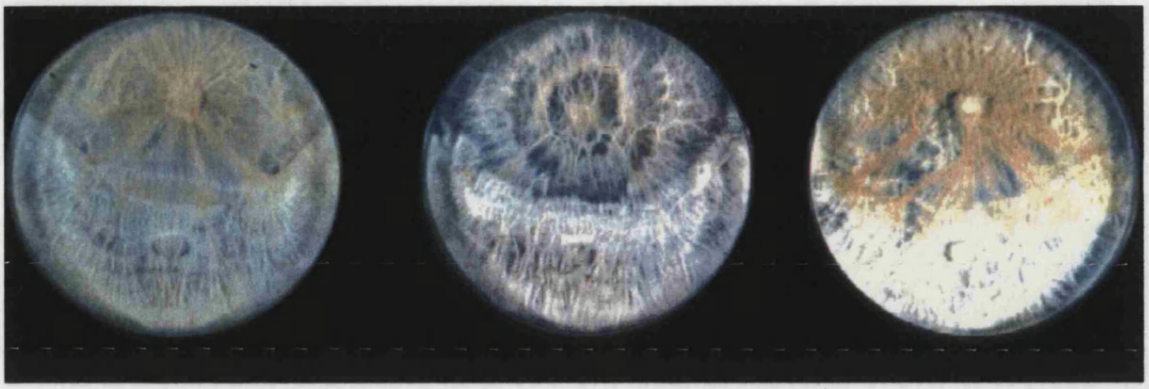
experiment. In interactions with fungus KB, droplets of reddish-brown pigmented fluid were exuded at the interface zone immediately after the mycelia made contact. As time progressed these droplets grew larger and pigmentation spread into the growth media, eventually forming a band between 1cm and 2cm wide at the interface between the two mycelia. After mycelial contact was made, *H.fasciculare* aerial mycelium formed low ridges at the interface with KB, these ridges then acted as foci for the subsequent formation of mycelial cords. Once the interaction zone had been bridged, these cords ramified across the surface of the KB colony, eventually covering it completely, but at a lower density than the cords invading *P.radiata*.

The Class 2 *H.fasciculare* dikaryons showed low combative scores against both antagonists compared to their Class 1 counterparts, but were much weaker against KB than *P.radiata*. In pairings with *P.radiata* the Class 2 dikaryons tended to invade small areas of *P. radiata* mycelium by means of replacement fronts; cord formation was generally absent. In pairings against KB the Class 2 dikaryons tended to produce deadlock reactions, with neither mycelium invading the others territory. Only one Class 2 dikaryon invaded KB territory with cords. Such reactions generated little pigmentation.

H.fasciculare homokaryons showed the lowest combative scores, with a negative score against KB. Against *P.radiata* these cultures tended to either form deadlock reactions or gain small amounts of territory by replacement fronts. When the same homokaryons were paired against KB they lost territory and were often overwhelmed by sheets of KB mycelium. There was little or no pigment production in any of the homokaryon/antagonist pairings.

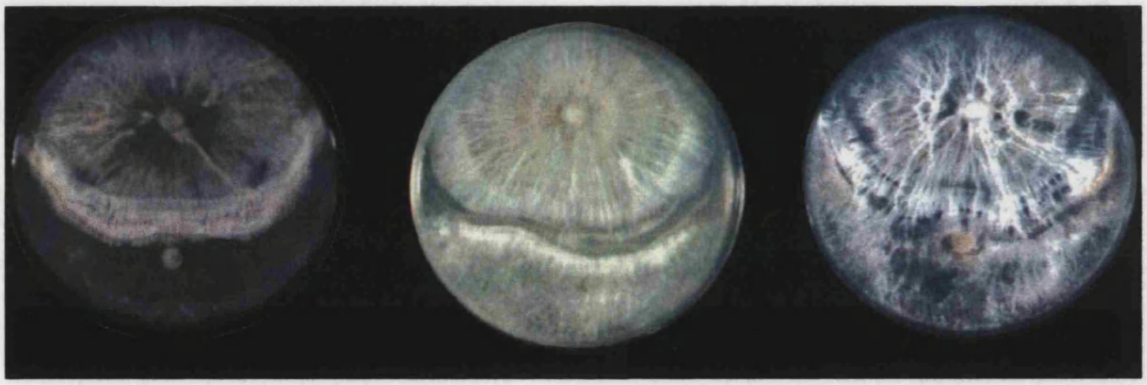
Figure 6.6 : Representative images of 76 day *H.fasciculare-P.radiata* pairings

Class 1 dikaryons vs *P. radiata*



S3C S7M AVS1F

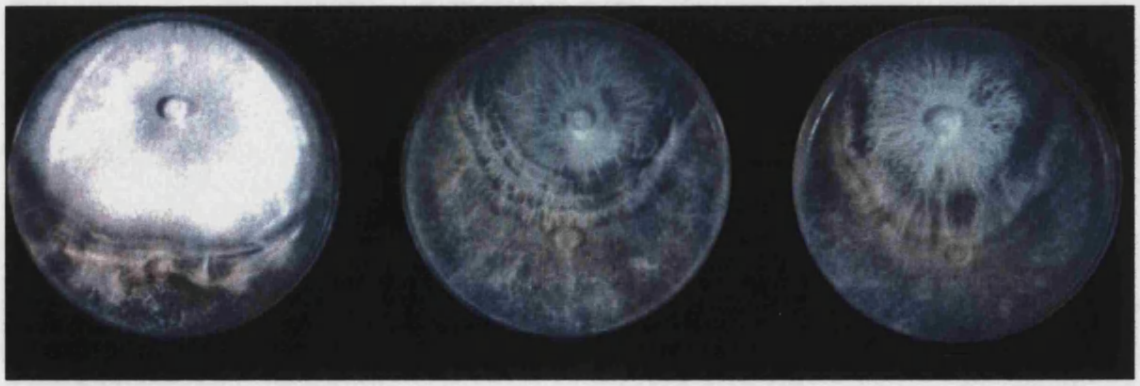
Class 2 dikaryons vs *P. radiata*



S2D* S6D AVS2B

*Image after 27 days; 76 day image not available

Homokaryons vs *P. radiata*

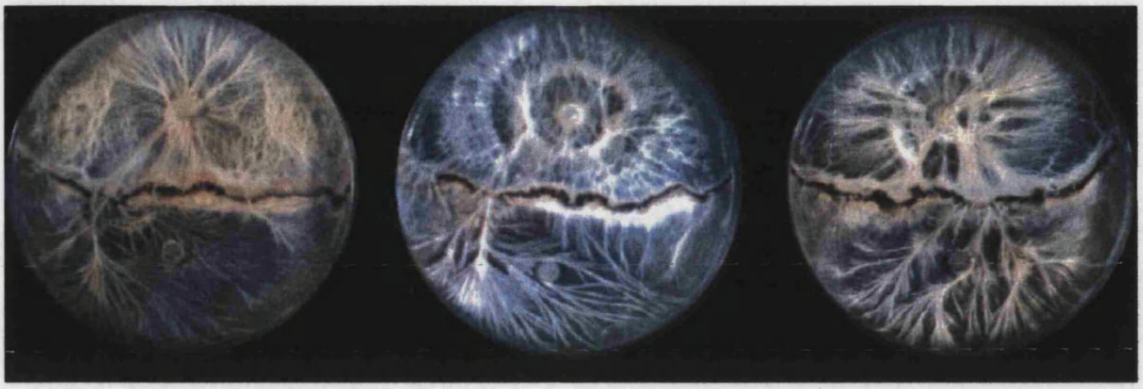


IAC-40 IIAC-84 AV-7

Note: The *H.fasciculare* inoculation point is at the top of each plate image.

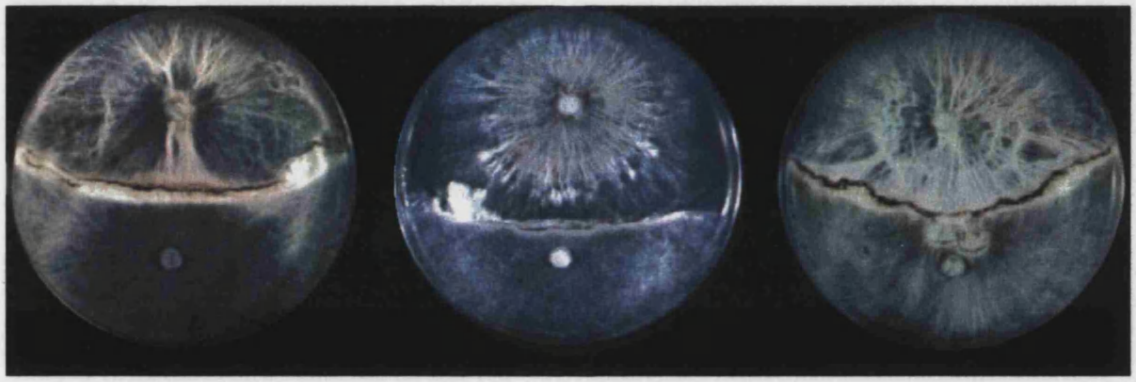
Figure 6.7 : Representative images of 76 day *H.fasciculare-KB* pairings

Class 1 dikaryons vs *KB*



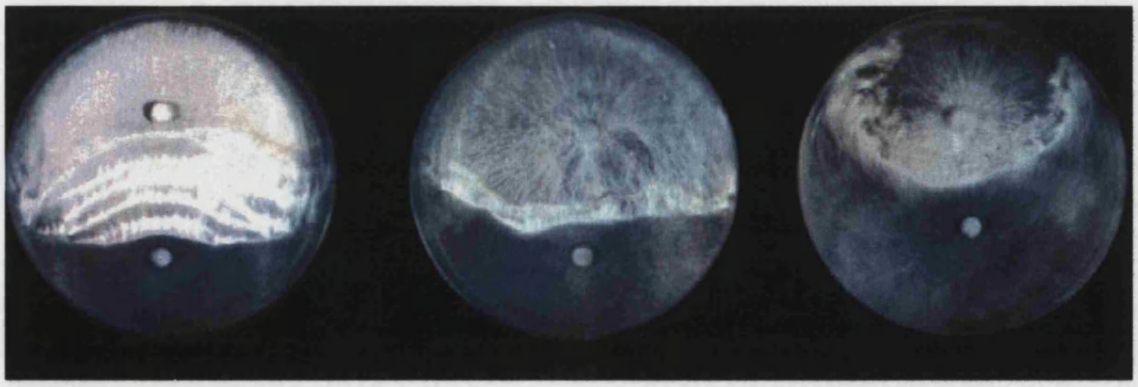
S3C S7M AVS1F

Class 2 dikaryons vs *KB*



S2D S6D AVS2B

Homokaryons vs *KB*



IAC-40 IIAC-84 AV-7

Note: The *H.fasciculare* inoculation point is at the top of each plate image.

The hierarchy of combative ability within *H.fasciculare* isolates derived from these experiments is Class 1 dikaryon > Class 2 dikaryon > Homokaryon. In each case KB proved to be a more resilient opponent than *P. radiata*. If this result is compared with the HPLC metabolite data in Chapter Five it can be seen that this hierarchy generally correlates with the ability of these three classes of *H.fasciculare* to produce hydrophobic metabolites.

In a previous study of interspecific reactions between *H.fasciculare* and other fungi, an overall reduction was found in the recovery of *H.fasciculare* metabolites from interactions as opposed to self-pairings (Griffith *et al* 1994*b*). This reduction was not evenly distributed between peaks Ha-Hg; the relatively hydrophilic peaks Ha and Hb increased in the interactions by up to several hundred-fold, whereas peak Hc decreased by 25-50% and the hydrophobic peaks He, Hf and Hg showed dramatic decreases of between one and three orders of magnitude. Peak Hg was the most reduced, shrinking to 0.1% of its pure-culture level in interactions with *P.radiata*. Although the simplest hypothesis; that the compounds were not produced, cannot be ruled out, there may be a more interesting possibility. This is that the metabolites were synthesised by the mycelium, but then immediately integrated into polymerised insulative material by the activity of extracellular phenoloxidase enzymes. A likely candidate enzyme is laccase, which has been found at high levels of activity in the interaction zones of *H.fasciculare* pairings. This would represent the switching of the mycelium into a more insulated, combative mode capable of resisting and counter-attacking the advances of *P.radiata*. Further evidence for this theory comes

Table 6.2a : Outcomes of *H.fasciculare* vs KB pairings after 76 days

H.fasciculare strains	Combat Score				
	-2	-1	0	+1	+2
Class 1a,b IAC				S3C	S1A S1C S1F S1H S1L
Class 1 IIAC					S4C S6D S7B S6C
Class 1 AV					1E 1F IG 3D
Class 2 IAC			S2D S1D		
Class 2 IIAC		S7C S7M			S7D1 S7H
Class 2 AV			2B 2K	1K2	
Homokaryon IAC	44	34 40 47			
Homokaryon IIAC		84 124 136 140			
Homokaryon AV		1 9 10	7		

Table 6.2b : Outcomes of *H.fasciculare* vs *Phlebia radiata* pairings after 76 days

H.fasciculare strains	Combat Score				
	-2	-1	0	+1	+2
Class 1a,b IAC				S3D	S1A S1C S1F S1H S1L S3C*
Class 1 IIAC					S4C S6C S6D S7B
Class 1 AV					1E 1F 1G 3D
Class 2 IAC				S1D S2D	
Class 2 IIAC			S7M	S7C	S7D1 S7H
Class 2 AV		2K		2B	1K2 3L*
Homokaryon IAC			34 40 47		44
Homokaryon IIAC				84 124 136 140	
Homokaryon AV			1	7 10	9

*Paired against *P.radiata* only

Scoring system

+2 *H.fasciculare* replaces antagonist

+1 *H.fasciculare* invades antagonist territory

0 Deadlock

+1 Antagonist invades *H.fasciculare* territory

+2 Antagonist replaces *H.fasciculare*

Table 6.3 : Mean combat scores for different *H.fasciculare* classes vs *Phlebia radiata* and fungus KB

H.fasciculare class	Mean score against *P. radiata*	Mean score against KB
Class 1 dikaryons	+ 1.9	+ 1.9
Class 2 dikaryons	+ 1.1	+ 0.3
Homokaryons	+ 0.8	- 1.0

from the fact that the addition of EDTA or CET, both specific laccase inhibitors, to the growth medium significantly eroded the combative potential of *H.fasciculare* in pairings with *P. radiata* or *Peniophora lycii*. (Griffith *et al* 1994*b*). Although the circumstantial evidence for this hypothesis is strong, proving it will require characterisation of the chemical structure of Hg, proof of the laccase/Hg catalytic interaction and ultrastructural study of *H.fasciculare* hyphal cell walls to localise the presumed insulative polymerised material.

Hydrophobic metabolites in H.fasciculare: General conclusions

The experiments described in this chapter add further to the circumstantial evidence that peaks He and Hg represent insulative metabolites important in mycelial morphogenesis.

HPLC analysis of liquid cultures of *H.fasciculare* show that He and Hg metabolites are associated with the mycelium and are not liberated into the culture medium. Further evidence may be drawn from the results in Chapter Five and the first part of this chapter; these show that Hg (and He to a lesser extent) is absent or aberrant in both homokaryons and the Class 2 'flat' dikaryon strains recovered from fruit-body tissue. Class 2 dikaryons have sharply reduced aerial mycelium, cord-

formation and combative ability compared to normal Class 1 dikaryons, while homokaryons do not form well-differentiated cords and have a very low combative potential.

Taken as a whole, the evidence for the importance of the He and Hg peaks is strongly suggestive but does not (yet) allow a chain of cause-and-effect to be established showing that these metabolites are prime determinants of morphology in *H.fasciculare*. They may simply be markers or aspects of other, underlying processes within the mycelium. The accumulated evidence points to a close association, if not identity, between these metabolites and important phenotypic processes, but biochemical characterisation of these compounds and their genetic and epigenetic control may be necessary before their importance can be verified.

If the idea of an 'insulative' role for the Hg metabolite(s) is pursued, there are interesting parallels between the appearance of the Hg peaks in *H.fasciculare* and the expression pattern of the hydrophobin genes in *Schizophyllum commune*. Hydrophobins are a family of short (approx. 100-amino acid) proteins with a high proportion of hydrophobic amino-acids and eight conserved cysteine residues. Hydrophobins are widely produced by filamentous fungi but have been best characterised in *S. commune* (Wessels, 1995). The main role of hydrophobins seems to be as hyphal wall components, particularly in aerial mycelia, fruit bodies and spores where they form densely packed 'rodlet' layers. Once assembled into such layers the hydrophobin molecules are extremely resistant to normal techniques of protein solubilisation, even though the bonding between them is thought to be non-covalent in nature.

The pattern of hydrophobin expression is controlled throughout the mycelium by the activity of the mating-type genes. Monokaryotic hyphae transcribed only hydrophobin SC3, whereas dikaryotic mycelium transcribed hydrophobins SC1, SC3, SC4 and SC6. Disruption of the close pairing of nuclei in the dikaryon, particularly in aerial mycelium, resulted in the reduction of dikaryon-specific genes and an increase in the proportion of SC3 transcription. Targeted mutation of the SC3 hydrophobin gene led to strains blocked in the production of aerial mycelium in sealed culture and only capable of producing wettable aerial mycelium in aerated cultures. Dikaryons mutant in both SC3 genes formed wettable aerial mycelium but could fruit normally (Wetter *et al* 1996).

Based on the HPLC and culture evidence, an analogy can be drawn between the dikaryon-specific hydrophobins of *Schizophyllum* (SC1, SC4 and SC6) and the Hg, and possibly He, metabolites of *H.fasciculare*. The Hg peak is present in the normal *H.fasciculare* dikaryon, but not in the homokaryon or the flat dikaryons. The SC1p,. SC4p and SC6p hydrophobins are present in the *Schizophyllum* dikaryon, but not in the homokaryon or in dikaryotic hyphae with disrupted nuclear association. Considering the ubiquity of the hydrophobin genes in the filamentous fungi, it would be reasonable to assume that *H.fasciculare* possesses its own hydrophobins. The polymerisation of Hg metabolites by laccase may represent an additional boundary-sealing system that supplements dikaryon-specific hydrophobins.

Chapter Seven

Analysis of *H.fasciculare* growth and differentiation in a heterogeneous culture system

Introduction

Heterogeneous culture systems

The strategies used for the study of fungal growth in heterogeneous environments can be split broadly into field studies and laboratory-based experiments. Field studies have the advantages and disadvantages of authentic environmental conditions. The disadvantages include difficulties in set-up, accessibility and reproducibility, but the advantage is that any results gained will have a high relevance to fungal growth in the real world. In some cases the environment has been brought into the laboratory in the form of soil-tray experiments (Dowson *et al* 1989b), where wood-block inocula and baits are distributed in non-sterile soil. This allows the processes of cord-formation and foraging to be studied in an accessible manner.

Many laboratory-based studies of fungi in heterogeneous media have been based on agar culture systems with the controlled addition of different amounts or types of nutrients (Amir *et al* 1994, Olsson 1995). Agar-based experiments have the advantage that mycelial growth occurs in a more-or-less transparent medium; this allows better quantification of the dynamics of the 'high-nutrient' phases of mycelial growth than wood-block experiments. It can also allow precise measurements to be made of nutrient distribution within the medium (Olsson, 1993).

The matrix-plate heterogeneous culture system

The matrix-plate is a culture-plate with a 'matrix' of interconnected compartments within which a mycelium can be grown on a controllable, varied pattern of media. The two most important control factors are the limited interconnections between the compartments and the distribution of different media amongst the compartments. The

interconnections are designed to allow fungal growth but not diffusion within the substrate. Using this system, both interconnection and media distribution are available for experimental manipulation. The interconnection of compartments provides a way of physically controlling the spread and differentiation of a fungus in culture. When a cord-forming fungus is inoculated into a matrix-plate, this has the effect of channeling cord formation along predictable paths within each compartment, giving a highly reproducible colony pattern of cord formation.The most open (and commonly used) pattern of interconnection is for each compartment to have one connecting channel to every adjacent neighbour compartment. The simple effects of a growth bottleneck and diffusion barrier between old and newly colonised compartments can have a profound effect on the growth mode and organisation of fungal colonies (Watkins, unpublished).

The design of the matrix-plate allows a fungal colony to grow across compartments containing different media without any 'blurring' due to diffusion effects within the media. The combination that has been used in this study is a simple high-nutrient/low-nutrient agar regime using 2% malt and distilled water agar, although many other permutations are possible including the distribution of chemical inhibitors or promotors, alternative growth substrates (such as glass beads) and even competitor organisms from the same or different fungal species.

Previous experiments using the matrix-plate technique have tended to use chequerboard patterns with fairly high levels of nutrient-rich agar. Cord-forming fungi in general, and *Hypholoma* in particular, have large organisational scales ranging from many centimetres to metres in their natural habitat. (Dowson *et al* 1988). The small-scale variation within a chequerboard matrix-plate may not be enough for such fungi to exhibit their full developmental potential. Matrix plate cultures can be designed to

simulate a larger-scale foraging situation, where a mycelium growing from an initial resource base encounters a large low-nutrient area containing a localised high-nutrient patch. Experiments such as this may yield information about mycelial responses additional to that recovered from than chequerboard design.

Water gradients and potentials within the matrix-plate system

An important factor in agar-based nutrient translocation experiments is the existence or formation of osmotic gradients, which can strongly affect nutrient translocation (Jennings, 1994). In the matrix-plate system detailed here there are two factors that give rise to such gradients; solute concentration and evaporation. The first is intrinsic in the WA/2MA culture system; as WA is simply 2MA without the malt extract the two media were not osmotically balanced. The straightforward diffusion effects of this were limited by the physical division of the plates; WA and 2MA only came into contact at very limited areas at the bottom of the connecting slots. However, a growing mycelium spans both 2MA and WA and is therefore subject to osmotic gradients. These gradients may well change during the course of the experiments as growth and metabolism of the mycelium alters the initial solute concentrations in WA and 2MA compartments.

The second factor affecting osmotic gradients is evaporation. Experience has shown that compartments at the edge of the plates dry out more rapidly than the central compartments; evaporation rates from agar are also dependent on the density of mycelial coverage. Colonised agar loses water more slowly than uncolonised agar; the thicker the aerial mycelium, the more pronounced the difference. As aerial mycelium is

often thicker in high-nutrient compartments, the low-nutrient compartments tend to dry out faster.

Analysis of heterogeneous cultures

Image Analysis of Fungal Colonies

Recent increases in the power and availability of computer-based image analysis systems have led to an similar increase in their use in biological research. Image analysis is essentially a toolbox of processing and analysis functions which can be used in several ways to extract information from digital images. Features of interest can be defined and then isolated from a background; dynamic processes can be analysed using a time-series of such images. Variation in image properties such as texture, brightness and contrast can also be quantified. Several factors need to be considered when attempting to use image analysis to gather data. The most important consideration is the biological significance of the data and how it relates to the biological processes under study. Image-analysis can provide huge amounts of data very quickly, but this can lead to confusion rather than insight.

One measurement which is relatively easy to acquire from a back-illuminated fungal culture is the average brightness or 'light-density' of an area, measured on a greyscale (often on a 0-255 scale). As the hyphae in a mycelium refract and absorb light, the brightness of an area of mycelium may be inversely correlated with its biomass. This excludes any effects of pigmentation and differences between aerial and substrate mycelia. Aerial mycelia are more refractile than substrate mycelia due to the larger difference between the air/cytoplasm refractive indices than the water/cytoplasm refractive indices. Since *H.fasciculare* dikaryons produce pigment as they age, the

measure of mean light density does not reflect biomass directly. This can be compensated for by using images of mature colonies, where it may be presumed that all parts of the mycelium have gone through most of their pigment-production phase and achieved a relatively stable light-density, composed from both pigmentation and mycelial biomass components. If pigmentation is proportional to biomass then it need not invalidate the measurement, although it does complicate it.

The measurement of the mean light density of a matrix-plate compartment makes the assumption that the mycelium within a compartment is relatively homogeneous. As colonising mycelium can enter a compartment from different directions and from different circumstances, this is not always the case. It might be possible in the future to clarify this by subdividing such compartments into different analysis zones.

HPLC metabolite profiles of matrix-plate cultures

HPLC analysis of *H.fasciculare* in homogeneous culture has already yielded information on inter-isolate differences (Chapters 5 and 6). One of the advantages of HPLC is its sensitivity; this allows the metabolites present in the small agar blocks from individual matrix-plate compartments to be analysed and compared. When combined with image-analysis techniques this may allow a detailed picture to be assembled of the physiology and biochemistry of different parts of the same mycelial network as it responds to a heterogeneous environment.

Methods

H.fasciculare strains used for matrix-plate experiments

Two AVS dikaryon strains (AVS 1F and AVS 3E) were selected for analysis in matrix-plate experiments. AVS 1F was a normal dikaryon strain that exhibited vigorous growth and aerial mycelium production. AVS 3E was a 'flat' dikaryon strain showing reduced growth rate and aerial mycelium production. Due to the natural role and very slow growth of the homokaryon there seemed to be little to gain from studying it using the matrix-plate system, so no attempts were made to do so. Translocation experiments on fungi with similar growth patterns to *H.fasciculare* homokaryons have been carried out using two-way split-plate methods (Olsson 1995) and this may be a more suitable system for analysing the homokaryon.

Matrix Plate Heterogeneous Culture System

Matrix-plates: Dimensions and method of use

Matrix-plates were adapted from multi-compartmented square culture plates. These consisted of 5 by 5 arrays of $2cm^2$ compartments. The plates were 1.75cm deep and were supplied with lids which could be placed in a 'vented' or a 'non-vented' orientation. In the 'vented' orientation, tabs on the dish and lid aligned to give a small air gap between the two. To convert these plates into matrix-plates, 2mm wide slots were cut 1cm down into the inter-compartment walls. The 3ml of solid media (2MA or WA) dispensed into each compartment filled the compartments up to the bottom of the slots, thus providing channels through which aerial mycelium could extend but blocking diffusion between compartments due to agar-agar contact.

The dispensing of media and the inoculation of *H.fasciculare* cultures into matrix plates was carried out as described in General Methods. The patterns of medium distribution and inoculation points are detailed below. After inoculation the lids were placed in the 'non-vented' alignment and the edges of the matrix plates were sealed with strips of paraffin film; this reduced the drying of the media and the entry of airborne contaminants. The matrix-plates were incubated at 20°C in the dark.

Image-analysis of *H.fasciculare* matrix-plate cultures

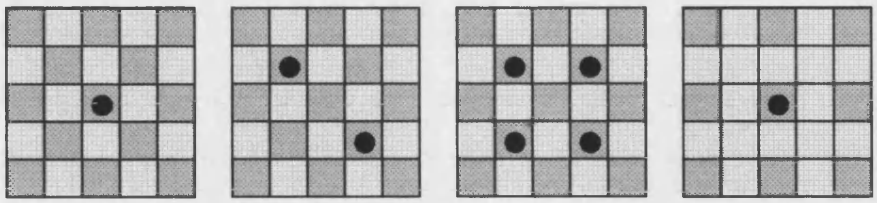
Image Capture Procedure

Digital images were captured from the matrix-plate cultures using a TMV-20 integrated imaging system (supplied by UVP Ltd) designed for the analysis of protein and DNA gels. The system included a mini-darkroom box with UV and white-light transilluminators and a monochrome CCD camera. This was connected to a 486 PC computer with an image-grabber card and GDS-8000 analysis software (UVP Ltd).

The matrix-plates were placed on the white-light transilluminator and digital images were acquired using the imaging system. The focus, zoom and aperture of the CCD camera were initially optimised, but were not adjusted further during the course of each imaging session. During time course experiments efforts were made to keep the exposure conditions the same for each time point, but this was hampered by the manual controls of the camera. After the cropping of extraneous background, 256-shade greyscale images of each matrix plate were acquired with approximate dimensions of 500 x 500 pixels.

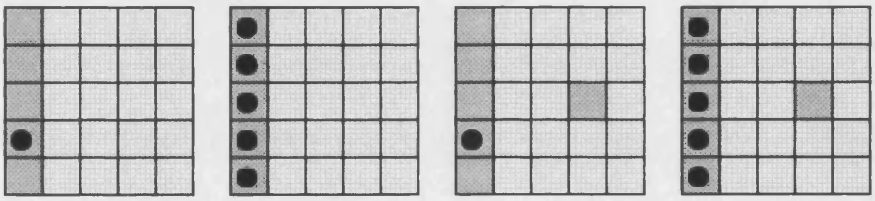
Figure 7.1 : Media distribution and inoculation of matrix plate experiments

Chequerboard-based matrix-plate designs

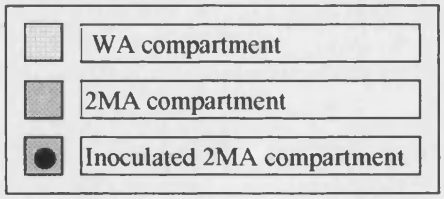


A B C D

Baited matrix-plate designs



E F G H



Time-course images of colony growth

The brightness of the time-course images was often variable, and this was corrected for by measuring the mean brightness of control regions of each image as described below, comparing the value with that of previous images, then adjusting the image brightness accordingly. The extension and differentiation of mycelia was highlighted in some cases by subtracting time-course digital images from each other, differences between the two images concerned showed up as dark areas on the output image. The quality of the subtraction images was variable due to difficulties aligning the input images exactly.

Quantitative Image Analysis Procedure

Statistical analysis: Mean light density

80 by 80 pixel squares corresponding to each matrix-plate compartment were cut from the original images using Paint Shop Pro v 4.10 (JASC Inc, 1996) image-handling software. These squares were statistically analysed using Visilog 4.1.5 (Noesis Ltd 1996) image-analysis software to give the mean and standard deviation of brightness levels for each square on a 0-255 greyscale, with white as 255 and black as 0. The means were converted to a negative scale by subtracting each one from 255, this gave a value for the darkness of the image with white as 0 and black as 255. This result was termed the 'mean light density' of the compartment. Mean light density was assumed to correlate with mycelial biomass, but no calibration experiments were performed in this case.

Control light density measurements of uncolonised 2MA and WA

As 2MA and WA media are different in colour, it was necessary to quantify the difference to aid in later comparison of mycelial growth density in the two types of media. Two matrix-plates images from the start of the time-course in Experiment One were used to provide data on the mean light density of uncolonised media.

HPLC analysis of matrix-plates

Extraction and HPLC of metabolites from matrix-plates

Ethyl acetate metabolite extraction and HPLC analysis of the colonised agar squares from matrix plates was carried out as described in General Methods.

Data analysis

The HPLC analysis reports from each compartment were used to provide three items of data; the area of the Hc peak, the area of the Hg peak and the total peak area for all metabolites. These were referenced to the media type and mean mycelial light density of each compartment (WA or 2MA).

Experimental design

Experiment One: Time-course of growth in flat and normal dikaryons

Aim: To investigate the dynamics of growth and differentiation in a normal and flat dikaryon.

Method: Normal (AVS 1F) and flat (AVS 3E) *H.fasciculare* dikaryon strains were each inoculated in six replicates of matrix-plate pattern **A**. The colonies were incubated

for eight days, then images of all the plates were captured at two-day intervals from day nine to day thirty-nine. (The day 25 time-point was missed due to an error.)

Experiment Two: 'Chequerboard' light density profiles

Aim: To study the final mycelial density profile and differentiation of a normal *H.fasciculare* dikaryon in various chequerboard-based matrix designs.

Method: Normal *H.fasciculare* strain AVS 1F was inoculated in matrix-plate patterns **A, B, C** and **D,** with three replicates made of each. A single set of images were captured after 55 days of growth.

Experiment Three: Light density profiles in large-scale 'foraging' situations

Aim: To study the final mycelial density profile and differentiation of a normal *H.fasciculare* dikaryon in comparatively large-scale foraging situations.

Method: Normal *H.fasciculare* strain AVS 1F was inoculated in matrix-plate patterns **E** and **F** (one replicate each) and **G** and **H** (two replicates each). A single set of images were captured after 55 days of growth.

Experiment Four: Metabolite/Light density comparison

Aim: To compare the mean light density and HPLC profiles of normal *H.fasciculare* dikaryon in two different matrix-plate designs.

Method: One pattern **A** and one pattern **H** matrix-plate (respectively from experiments Two and Three) were used for compartment-by-compartment metabolite extraction and HPLC analysis after 55 days. Comparisons were made between the metabolite data and 55-day mean light density for each compartment.

Growth and differentiation in Class 1 and Class 2 dikaryons grown in matrix-plates

Representative images of *H.fasciculare* Class 1 and Class 2 dikaryon growth in an A-design matrix-plate are shown below in **Figure 7.2**. The Class 1 dikaryon showed a regular, well-developed cord system and dense production of aerial mycelium in the 2MA compartments, particularly those on the periphery of the plate. The Class 2 dikaryon showed some cord development, but this was generally less ordered and finer in structure than that of the Class 1 dikaryon. As expected, the overall density of growth was lower in the Class 2 dikaryon.

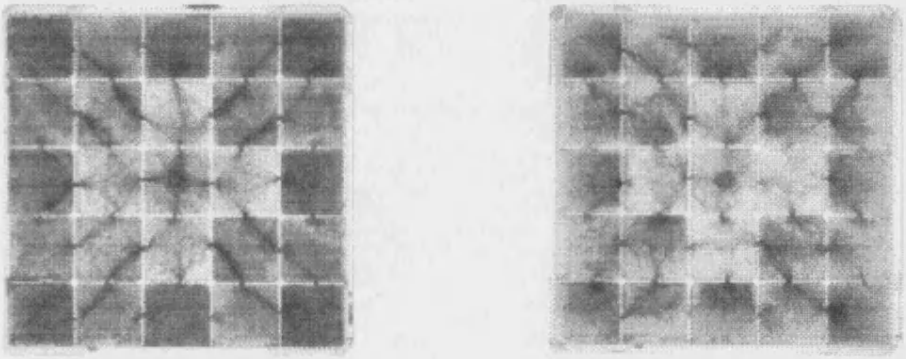


Figure 7.2: A Class 1 dikaryon (AVS 1F, left) and a Class 2 dikaryon (AVS 3E, right) after 36 days growth in a Design A matrix-plate (Back-illuminated)

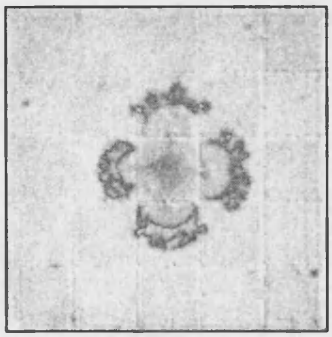
The pattern of mycelial density in response to the WA/2MA media distribution within the plates were similar in both cases; mycelium in 2MA squares was darker than mycelium in WA squares even after the difference between the media had been taken into account by comparison with uncolonised plates (**Table 7.1**). This difference was due both to higher aerial mycelium production and pigmentation in the 2MA squares.

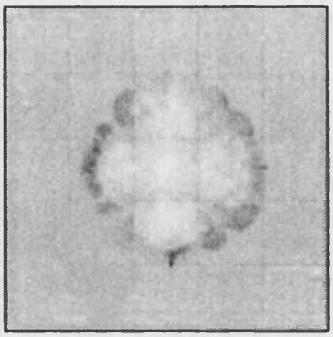
In both plates it can be seen that compartments on the periphery of the plates were darker than those in the centre. This pattern was stable upon long periods of incubation and so does not represent a progressive lightening of older mycelium. The pattern may represent either centripetal redistribution of resources within the mycelium or an edge-effect artefact of the culture system.

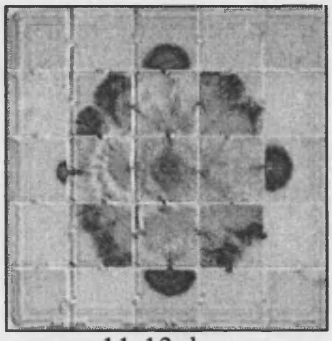
The time-series imaging of mycelial growth in the Experiment One matrix-plates produced data showing differences in the growth dynamics between a Class 1 (AVS 1F) and a Class 2 (AVS 3E) dikaryon. Two sets of results are shown in **Figure 7.3** and **7.4**; other replicates gave similar patterns. In these subtraction images the regions of mycelium that have grown during a time interval show up as dark zones, whereas areas of mycelium and media that have not changed are shades of pale grey. In the first 8 images of **Figure 7.3** an expanding dark ring can be seen; this shows that the entire periphery of the Class 1 mycelium has a relatively uniform extension rate within the constraints of the matrix plate. Although the mycelium is growing across a heterogeneous medium, there do not seem to be any obvious differences in mycelial extension rate between areas of mycelium in the 2MA and WA compartments. The corresponding set of images in **Figure 7.4** show that the Class 2 dikaryon has an irregular, 'stop-start' growth pattern, where different parts of the mycelium extend, stall for a period of a few days, then resume growth. This pattern does not seem to involve a regular 'pulsing' of growth within the mycelium.

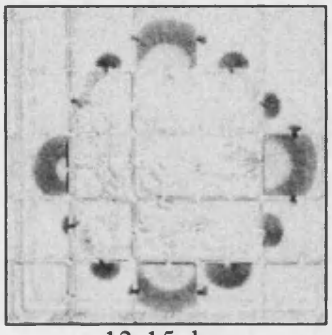
The last image in both figures shows the changes in the mycelia from day 25 to day 37. In **Figure 7.3** it can be seen that there is a slight increase in overall light density in all the 2MA compartments colonised by the Class 1 dikaryon during this period. This probably represents a small increase in pigmentation or proliferation

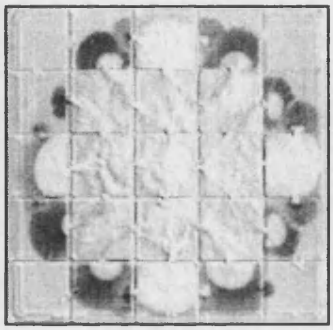
Figure 7.3 : Subtraction images of colony development of a Class 1 *H.fasciculare* dikaryon (AVS 1F) in an A-design matrix-plate

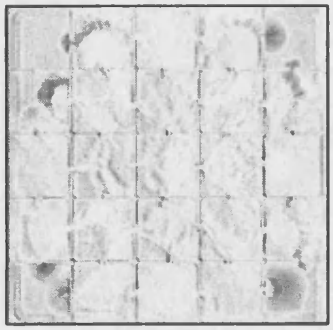

7-9 days

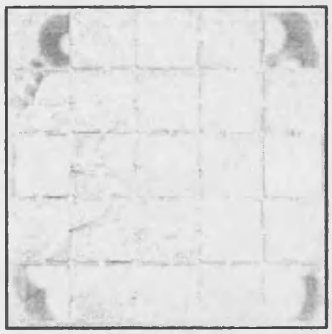

9-11 days

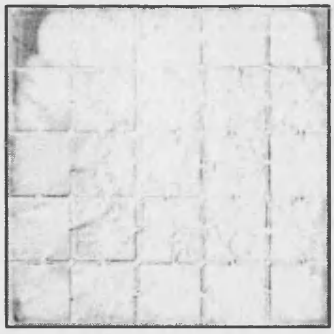

11-13 days


13-15 days


15-17 days


17-19 days


19-21 days


21-25 days

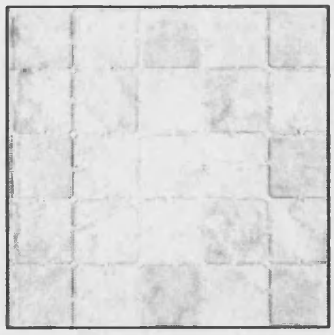
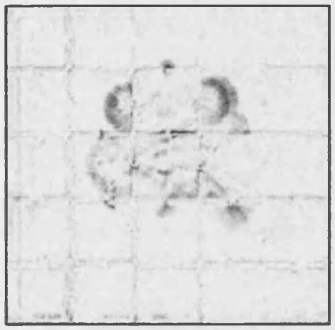

25-37 days

Figure 7.4 : Subtraction images of colony development of a Class 2 *H.fasciculare* dikaryon (AVS 1E) in an A-design matrix-plate

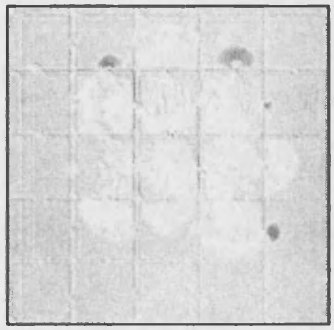

7-9 days


9-11 days

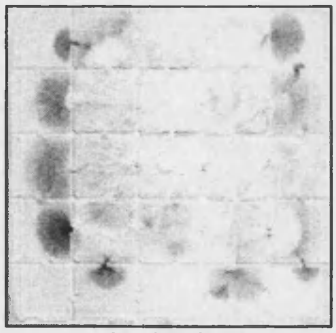

11-13 days

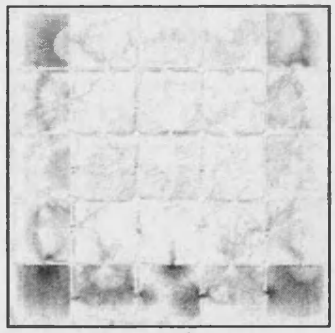

13-15 days


15-17 days


17-19 days


19-21 days


21-25 days

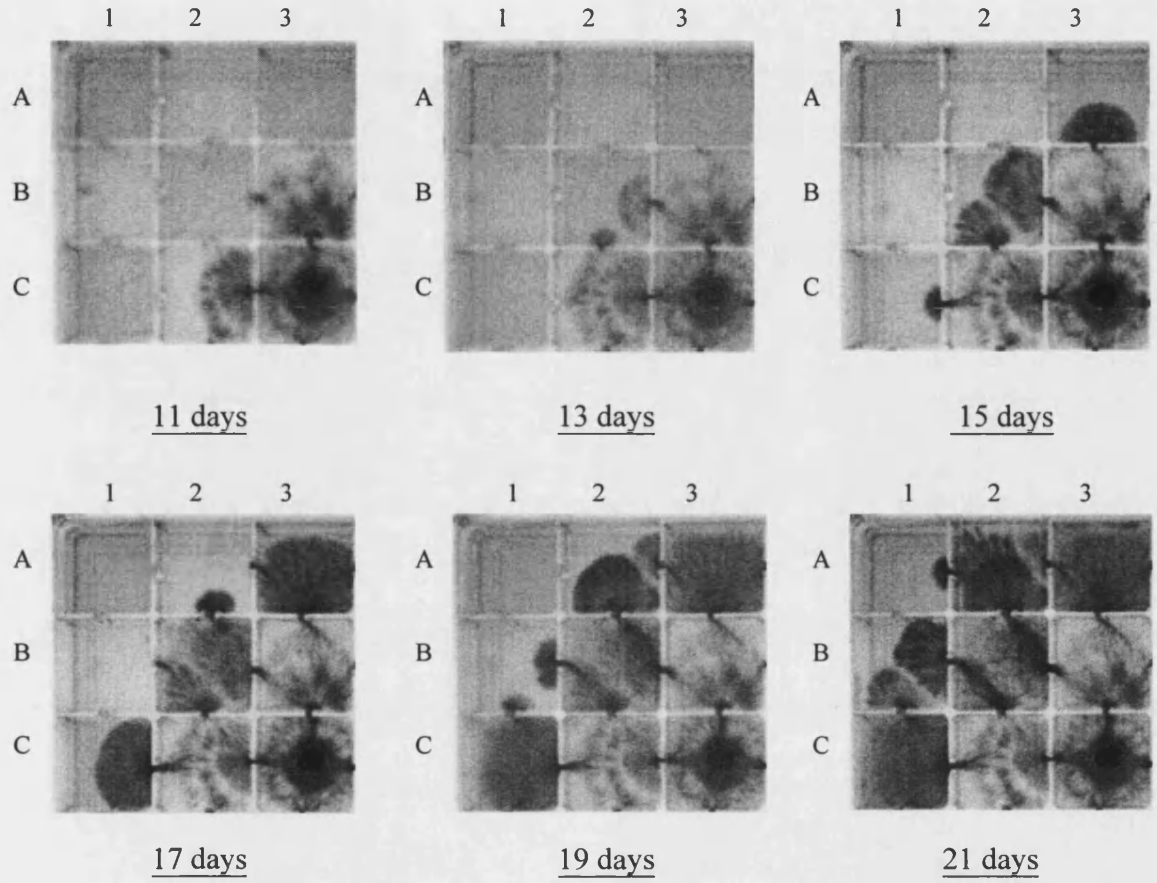

25-37 days

of aerial mycelium in these compartments. There is no corresponding increase in the 2MA compartments colonised by the Class 2 dikaryon, which produced sparse, unpigmented aerial mycelium in all compartments.

Representative time-sequences of cord formation in A-design matrix-plates colonised by the Class 1 and Class 2 dikaryons are shown in **Figures 7.5** and **7.6**, along with tables of colonisation and differentiation events. The process of cord formation followed a similar pattern in both Class 1 and Class 2 dikaryons, although Class 2 dikaryons took longer to complete the process. In both cases, primary cord formation was initiated when explorative mycelium from a compartment started to colonise an adjacent compartment. Reinforcement of the primary cords and the production of fine secondary cords occurred as this process was completed. No further changes in cord pattern were observed during the rest of the experimental period.

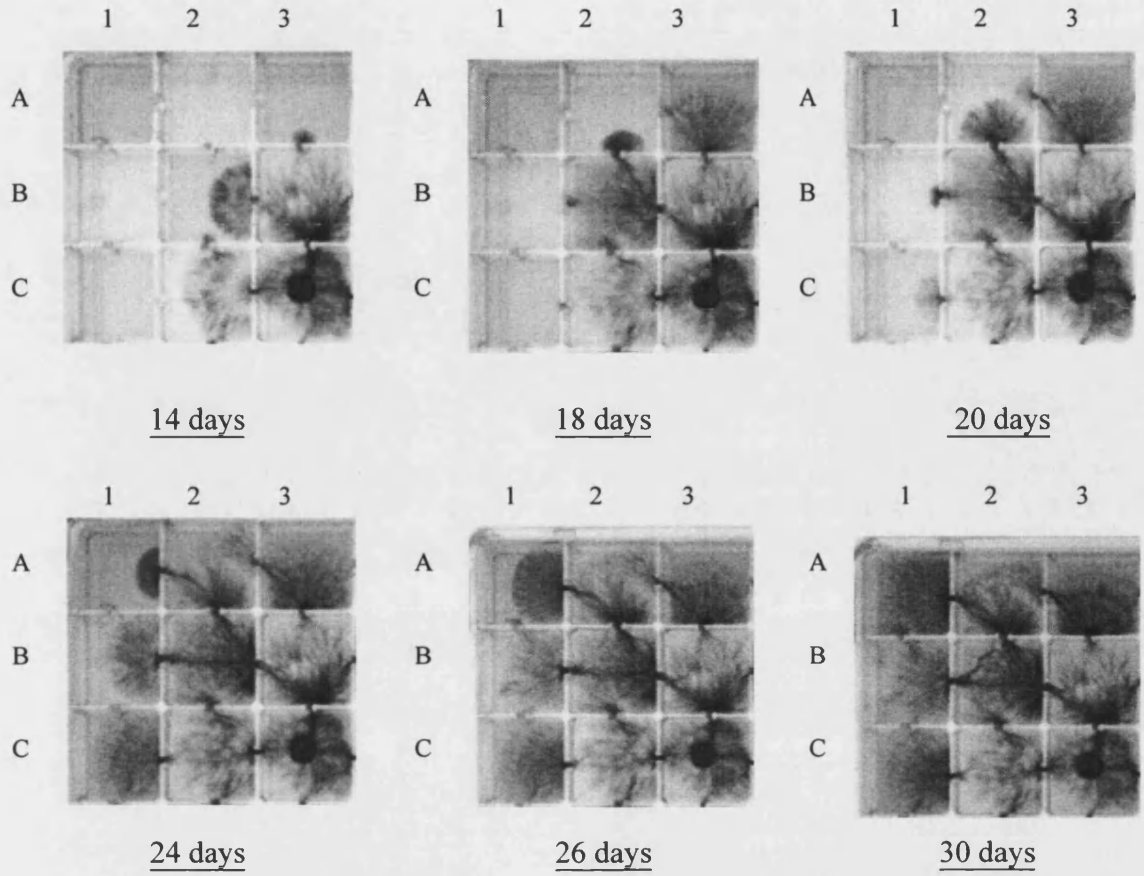
Mycelial cords are differentiated translocation organs, and the timing of cord differentiation indicates that cords forming behind the peripheral growth zone may be responsible for the translocation of resources to the edge of the colony and form in response to the extension of hyphae in these regions. In field and soil -tray experiments using non-sterile soil *H.fasciculare* dikaryons extend as fine cords (Bolton and Boddy 1993), although this is less evident in agar cultures. Close examination of mycelium colonising new compartments showed the presence of faint hyphal aggregates, which may be 'pre-cord' structures within the mycelium. The subsequent differentiation of cords may represent the selection and reinforcement of the few pre-existing hyphal aggregates that were successful in finding the exit to a new compartment.

Figure 7.5 : Time-course images of cord development by normal *H.fasciculare* dikaryon (AVS 1F) in 9 cells of a matrix-plate (Design A)



Time	Colonisation and differentiation events
11 days:	2MA compartment **2B** is uncolonised
13 days:	Diffuse exploratory mycelium enters **2B**
15 days:	Two fans of mycelium separated by a demarcation zone colonise **2B**
17 days:	The first exploratory mycelium emerges from **2B** to **2A**
19 days:	Colonisation of **2A** and **1B** (WA compartments) continues with the simultaneous formation of cords within **2B**
21 days:	Cord differentiation within **2B** is complete; no further changes seen.

Figure 7.6 : Time-course images of cord development by 'flat' *H.fasciculare* dikaryon (AVS 3E) in 9 cells of a matrix-plate (Design A)



Time	Colonisation and differentiation events
14 days:	Primary colonisation of 2MA compartment **B2**
18 days:	Colonisation of WA compartment **A2** initiates a cord in **B2**
20 days:	Further cord development links to **A2**; WA compartment **B1** is colonised
24 days:	Colonisation of **B1** initiates another cord in **B2**
26 days:	Cords in **B2** are consolidated
30 days:	A secondary cord develops across the top left corner of **B2**. No further changes are observed.

Effects of nutrient distribution and inoculation pattern on mycelial density profiles

The simplest models of mycelial density in matrix-plates can based on extreme assumptions about the integration of the fungal mycelium. If mycelial connectivity is zero, and hyphae grow and differentiate as 'hyphal growth units' isolated from the rest of the mycelium (Prosser, 1994) , then it may be predicted that the mycelial density in each compartment will depend totally on the nutrient supply within that compartment. If mycelial connectivity is total, the mycelial density in all the matrix-plate compartments should be dependant on the average supply of nutrient within the matrix-plate; the mycelium should 'smooth' the heterogeneity present in its environment.

An examination of the matrix-plate images in **Figure 7.1** indicates that the reality of growth within a matrix-plate is likely to lie somewhere between these two extremes of connectivity. A model was therefore derived that combined features of both. The data on the dynamics of cord differentiation indicated that the period of highest translocational activity was during the colonisation of a new compartment by explorative mycelium. From this observation the simplifying assumption was made that the primary direction of resource translocation during growth in chequerboard matrix-plates was centripetal and only occurred between adjacent compartments during colonisation. Each compartment was considered to be an input/output system, with colonising mycelium entering a matrix-plate compartment representing input and explorative mycelium leaving the compartment representing output. The balance between these processes was assessed for each compartment by examining the pattern of mycelial colonisation within the matrix-plate and giving a score of +1 for mycelial

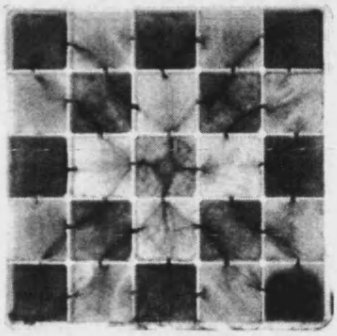
Figure 7.7: Mycelial flow maps and representative images of colonised matrix-plates

Matrix-plate design A

Flow map

2	1	-1	1	2
1	0	-3	0	1
-1	-3	-4	-3	-1
1	0	-3	0	1
2	1	-1	1	2

Matrix plate

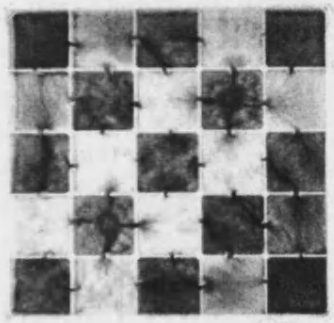


Matrix-plate design B

Flow map

2	1	1	-1	2
1	0	-2	-4	-1
1	-2	4	-2	1
-1	-4	-2	0	1
2	-1	1	1	2

Matrix plate

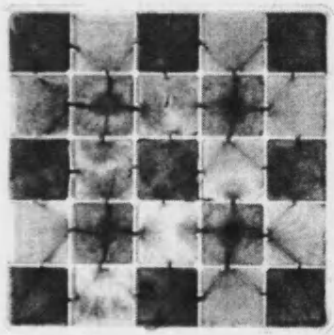


Matrix-plate design C

Flow map

2	-1	3	-1	2
-1	-4	0	-4	-1
3	0	4	0	3
-1	-4	0	-4	-1
2	-1	3	-1	2

Matrix plate



colonisation into a compartment and -1 for mycelial exploration out of a compartment. These scores were added up for each compartment to give a number termed the 'flow sum', the record of these numbers for a whole petri-dish was termed a 'flow map'. As an example, a matrix-plate compartment colonised through 1 connection and exploring outwards through 3 would have a flow sum of -2. The flow maps derived for matrix-plates of design A, B and C are presented in **Figure 7.7**, along with representative images of each plate design after colonisation by Class 1 dikaryon. The predictions made from the flow map model were that compartments with a negative flow sum value would be those acting as net sinks for mycelial resources; these would tend to be lighter than compartments with a positive flow sum value, which would act as net sources of mycelial resources.

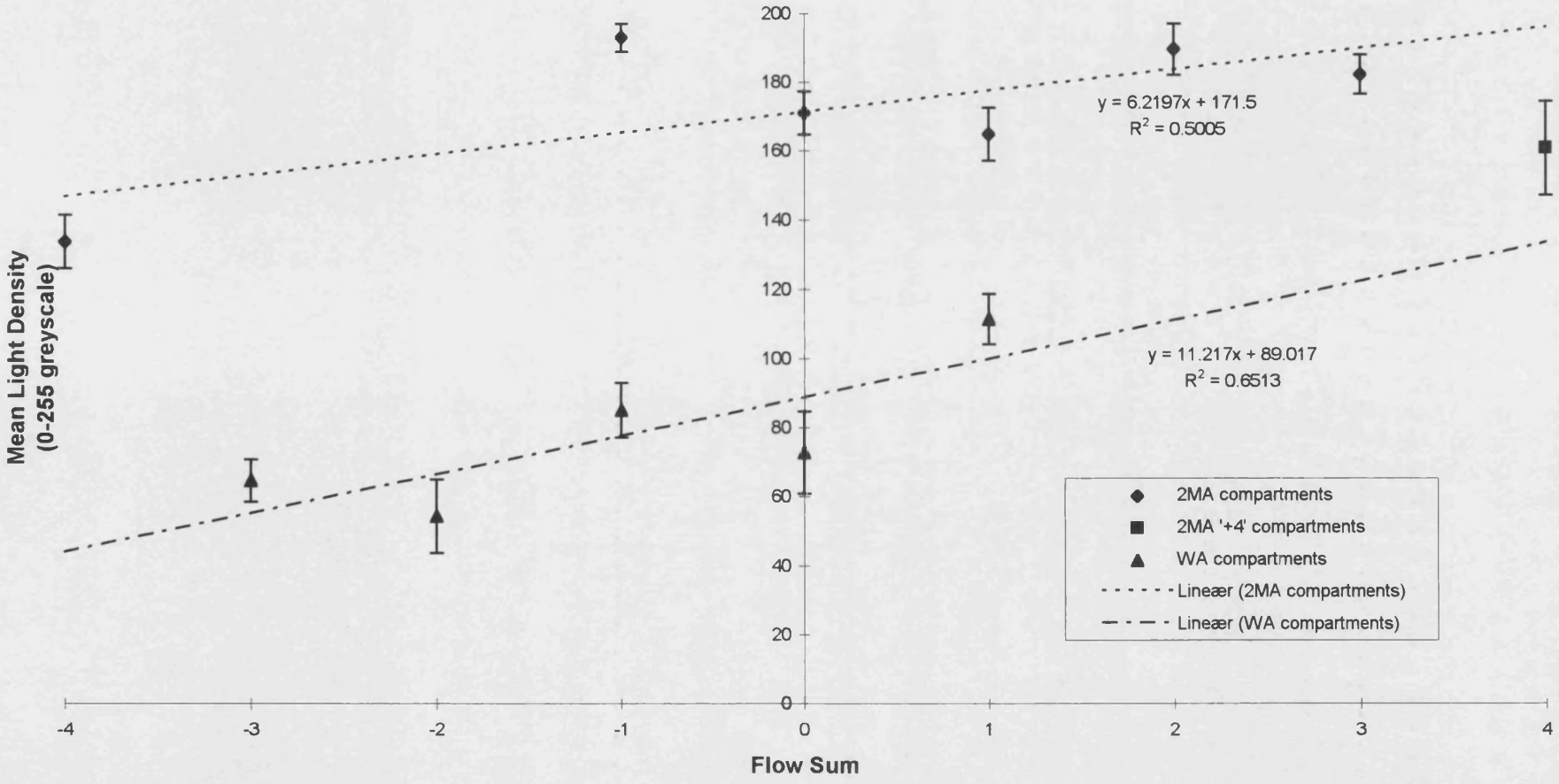
The compartment light densities were measured and compared to the flow sum values derived from matrix-plates designs A, B, and C. The design of these plates was intended to give a variety of growth patterns in an attempt to compensate for edge-effects. The WA and 2MA compartments were considered separately to account for variations in mycelial density due to the local nutrient supply. The initial difference in shade between 2MA and WA media was compensated for by measuring the greyscale difference and adding it to the WA measurement (***Table 7.1***).

Table 7.1 : Control light density measurements of uncolonised 2MA and WA medium

Uncolonised Plate	Mean Positive Greyscale	95% confidence limits	Mean Difference (2MA-WA)
Plate 1: WA	57.5	± 6.2	
Plate 1: 2MA	77.3	± 7.4	19.8
Plate 2: WA	116.9	± 4.2	
Plate 2: 2MA	130.0	± 4.9	13.1
Mean adjustment value applied to WA squares :			**+16.5**

The results are shown in **Figure 7.8**, where a positive correlation can be seen between flow sum and the light density in both 2MA and WA compartments. Compartments with a flow sum of +4 were not included in this measurement as the inoculation of these compartments was too heavy for the development of a fully integrated mycelium. This led to the development of pale demarcation zones between the colonising mycelial fronts within these compartments. The results in **Figure 7.8** show that the mycelium growing through the heterogeneous pattern of resources in a chequerboard matrix-plate responds both to local nutrient supply and to the demands of adjacent compartments. The local response to nutrient conditions is demonstrated by the different levels of mycelial density between 2MA and WA compartments after the difference in shade between the two media has been compensated for. The importance of adjacent compartments in determining the growth pattern within a compartment is demonstrated by the positive correlation between flow sum and mycelial density.

Figure 7.8 : Corrected mean light density *vs* flow sum (*with linear regression of light density against flow sum and 95% confidence limits*) for 2MA and WA compartments in design A, B and C matrix-plates (55 days)



Comparison of colonisation patterns in design A and design D matrix-plates

A comparison was made between the mean greyscale darkness of three design A and three design D matrix-plates. The difference between these was that design D had four fewer 2MA squares than the chequerboard pattern of design A; both designs had one central inoculation point (**Figure 7.1**). This enabled two mycelia with identical flow-map patterns but different patterns of nutrient allocation to be compared. The results are shown in ***Table 7.2***

+9	+0	+43	+35	+17
+36	+142	+5	+138	+30
+42	+4	+15	-4	+32
+16	+147	+13	+145	+36
+24	+34	+50	+29	+29

Table 7.2 : Mean mycelial density (3 replicates): Subtraction of matrix-plate design D values from design A values

As would be expected, the four 2MA compartments that are present in design A but absent in design D show a large difference in mycelial light density. However, there is also a positive difference in the majority of the other compartments, indicating that the mycelium is 'averaging out' the nutrient supply across the matrix plate. This includes the central inoculation compartment and the corner compartments, none of which are adjacent to the 2MA compartments switched between the two designs.

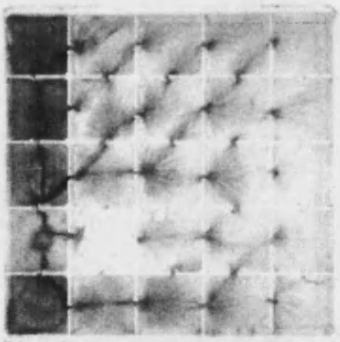
Colonisation pattern of 'foraging-pattern' matrix plates

The foraging-pattern matrix-plate designs E-H were intended to simulate larger-scale foraging situations than those possible with the chequerboard-based designs A-D. The growth patterns of the mycelia in these plates are shown in **Figure 7.9.**

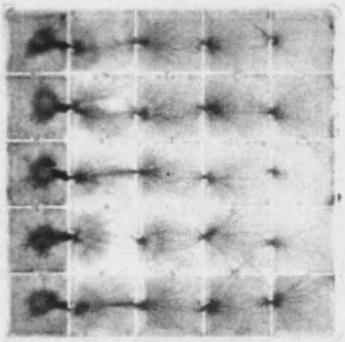
In the G and H design matrix-plates it can be seen that the 'bait' 2MA compartment has effects on many of the WA compartments surrounding it when these are compared to corresponding compartments in the unbaited E and F designs. Mycelium emerging from the 'bait' 2MA compartment at the right of the plates has a greater extension rate than the surrounding mycelium, which connected to the more distant resource base of the inoculum 2MA compartments. This demonstrates that *H.fasciculare* mycelial extension rate is at least partially dependant on the distance from the resource base. This information could not be obtained from chequerboard-design matrix plates as all low-nutrient compartments are adjacent to high-nutrient compartments; the distance to the nearest resource base is therefore constant throughout the plate. The second feature of the G and H designs is that translocation has occurred backwards from the 2MA 'bait' compartment into the WA compartment that colonised it. This WA compartment has visibly denser mycelium than the other WA compartments at the same distance from the inoculation site. The active redistribution of resources can therefore be seen in both directions from a colonised 2MA bait compartment.

As with the previous set of matrix-plates, flow-maps were made and compared with the mycelial density in the different compartments of each matrix-plate. These data are presented in **Figure 7.10**. Due to the design of the plates there was a less even distribution of flow sum values and fewer 2MA compared to WA compartments.

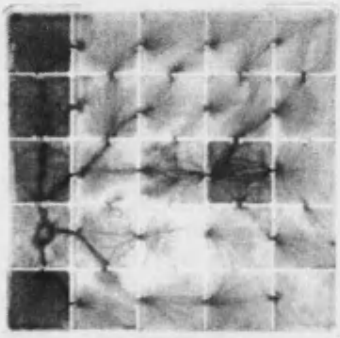
Figure 7.9 : Growth patterns of a Class 1 dikaryon (AVS 1F) in 'forage-pattern' matrix-plates designs E, F, G and H (55 days)



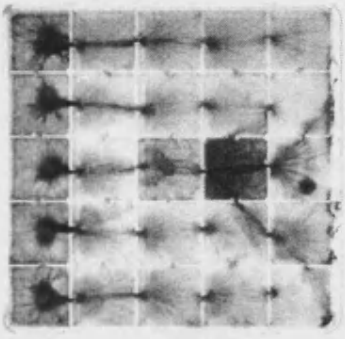
Design E (No bait)



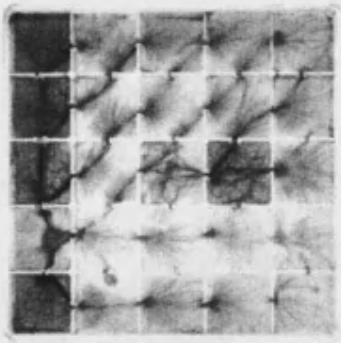
Design F (No bait)



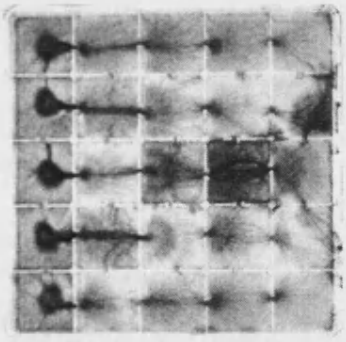
Design G (baited)



Design H (baited)



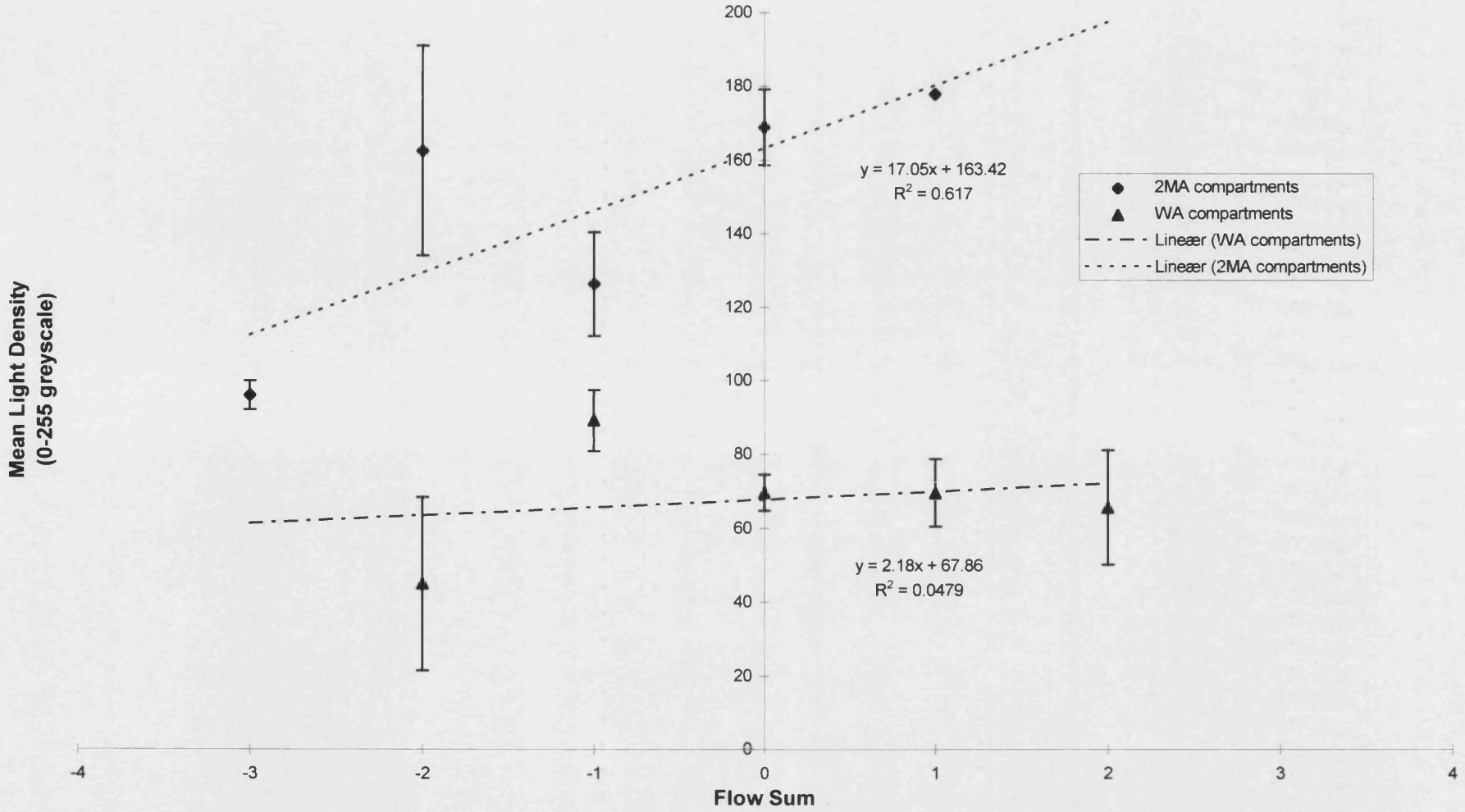
Design G (baited)



Design H (baited)

When compared to the data for the A, B and C designs in **Figure 7.8** it can be seen that the correlation between flow sum and mycelial density is positive for 2MA compartments, but that a similar correlation is lacking for the WA compartments. Two factors may be undermining the flow-map model for these compartments. The first is that basipetal translocation of resources to some WA compartments occurs from the 2MA 'baits'; this process is not accounted for in the flow-map model. The second factor is that mycelium in WA compartments at a distance from a 2MA resource base may not have spare resources to translocate outwards to newly colonised WA compartments. This mycelium may be 'subsisting' using very low levels of local nutrients and resource recycling processes.

Figure 7.10 : Corrected mean light density vs flow sum (*with linear regression of light density against flow sum and 95% confidence limits*) for 2MA and WA compartments in design E,F,G and H matrix-plates (55 days)



Production and distribution of metabolites within matrix-plates

Two matrix-plates, one of design A and one of design H, were harvested for HPLC metabolite analysis after 55 days. Peak Hc was visible in HPLC traces of from all the matrix-plate compartments; peak Hg was visible in about 50% of the traces. Some other small peaks were present but were not easily identifiable as the data were close to the resolving power of the HPLC system.

The total metabolite recovery for each compartment was compared to the mycelial density data for each matrix-plate. In **Figures 7.11** and **7.12** it can be seen that there is an approximately linear positive correlation between the mycelial density and the total amount of metabolites recorded by HPLC analysis. This may indicate a relatively straightforward, linear relationship between mycelial biomass and free metabolite production, although biomass has not been calibrated with mycelial light density. As the metabolite preparations from *H.fasciculare* were coloured (yellow/orange) it is possible that some of these metabolites are contributing directly to the pigmentation of the mycelium and hence the measurement of mycelial density.

Figure 7.11 Total metabolite peak area plotted against corrected mean light density with linear regression for 2MA (■) and WA (◆) compartments in a design A matrix-plate (55 days)

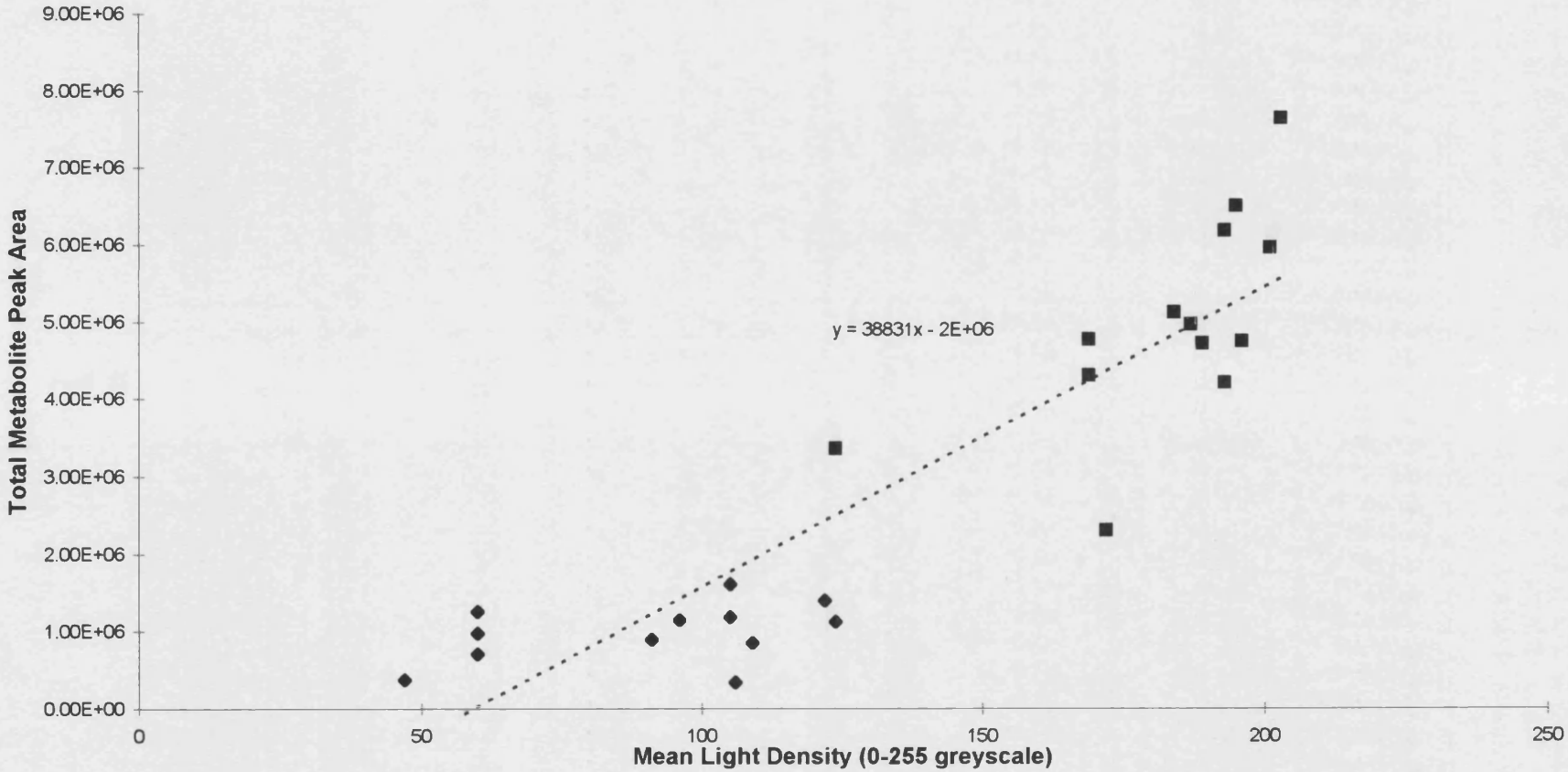
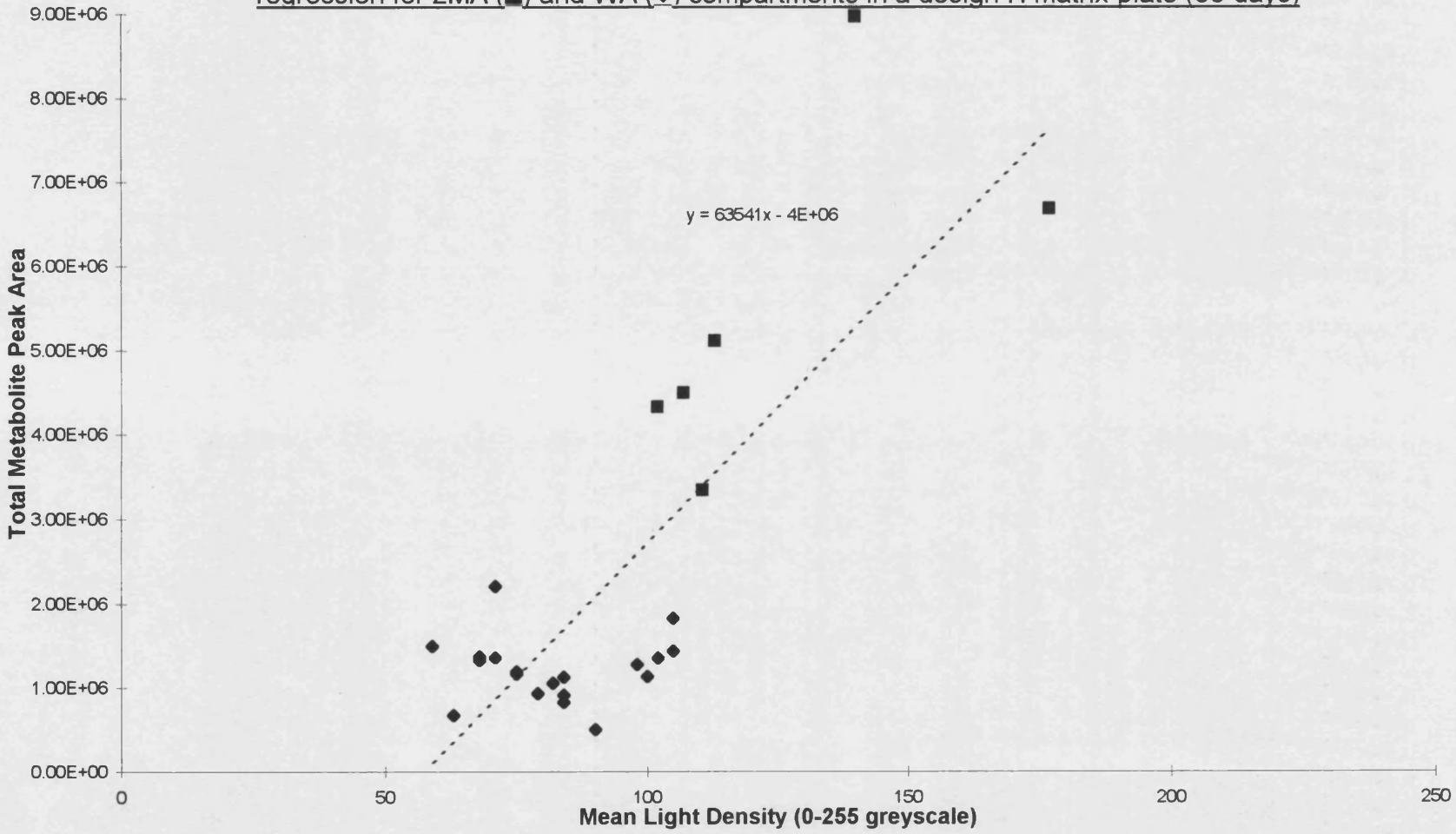


Figure 7.12 Total metabolite peak area plotted against corrected mean light density with linear regression for 2MA (■) and WA (◆) compartments in a design H matrix-plate (55 days)



Proportions of Hc and Hg in different matrix-plate compartments

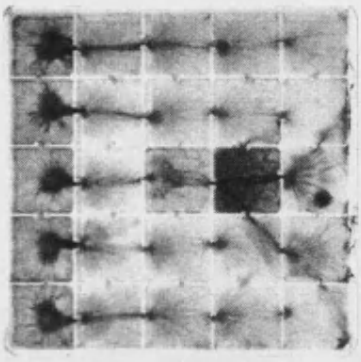
The levels of Hc and Hg in 2MA and WA compartments are illustrated in **Figure 7.13** and the mean levels compared in **Figure 7.14**. It can be seen that both metabolite peaks are preferentially expressed in 2MA compartments, but that the degree of difference is greater for Hg (20-fold difference) than Hc (5-fold difference). The yield of both metabolites is approximately 30% higher in the A-design matrix-plate than the H-design matrix-plate; this may either be a function of the relative age of the mycelium in each compartment or a result of nutrient redistribution. The design H plate has a multiple inoculation design and therefore a higher proportion of older compartments than the single-inoculation design A plate; if the amount of extractable metabolites in each plate starts to decay after a certain time then this could explain the lower metabolite recovery from the design H plate. Such decay has been tentatively established for peak Hc (Chapter Five, also Griffith *et al* 1994*b*) but not for peak Hg; the reduction seen in Hg in the design H plate therefore runs counter to this theory. The second possible explanation is that translocational effects have occurred within the mycelium that have tended to even out the available resources. Since design H plates have a lower proportion of 2MA compartments they will have a lower mean supply of nutrients, and a reduced output of metabolites may result.

Distribution of hydrophobic metabolites in matrix-plates

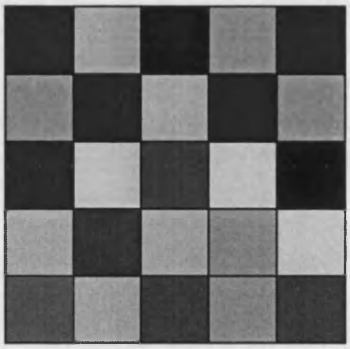
The Hg hydrophobic metabolites were seen in extracts from 2MA-grown colonies and in 2MA-compartments in matrix-plate experiments. Since an insulative role was predicted for such metabolites in Chapters Five and Six, and such a role should be correlated with low-nutrient conditions, it is reasonable to ask why the Hg metabolites

Figure 7.13 : Metabolite peak distribution within 55-day matrix-plate cultures

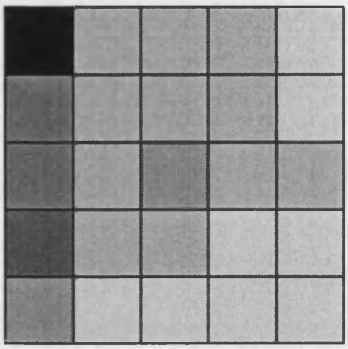
Matrix A plate



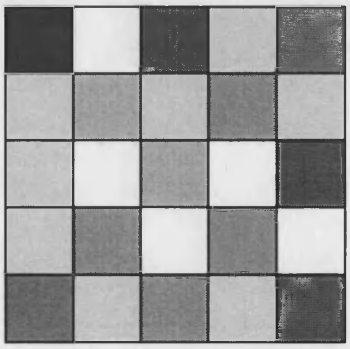
Matrix H plate



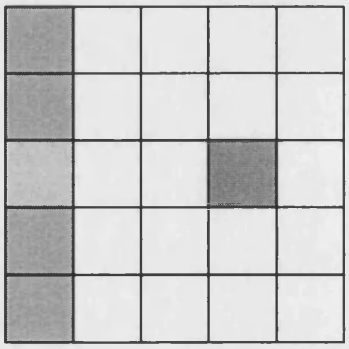
Peak Hc: Matrix A



Peak Hc: Matrix H



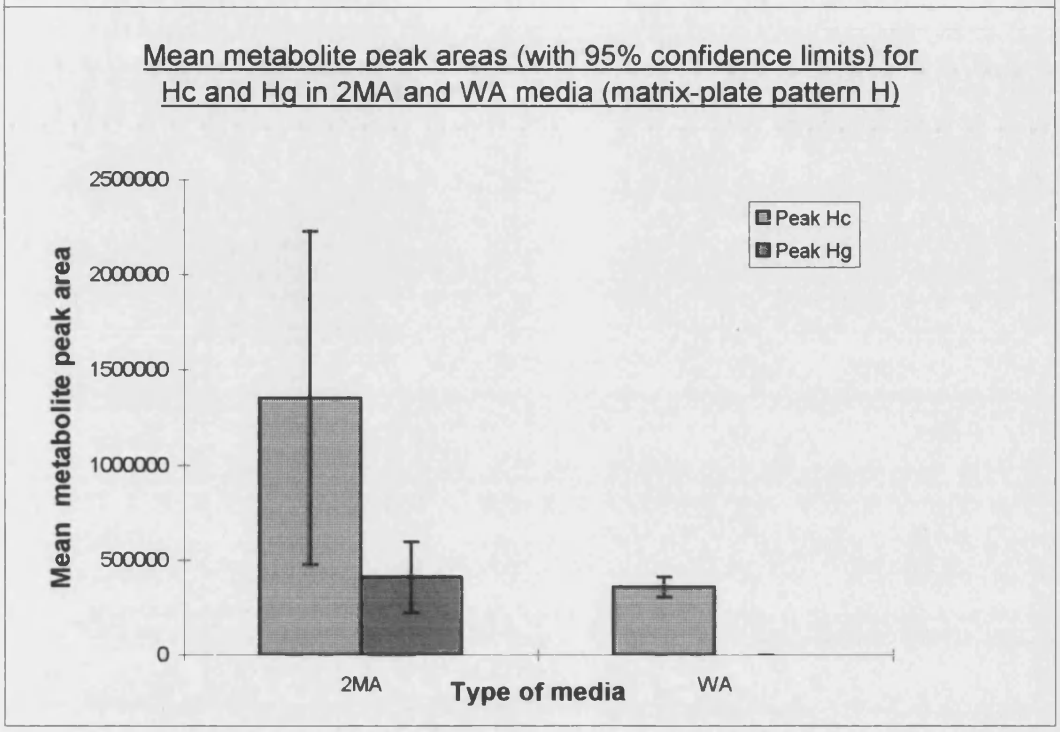
Peak Hg: Matrix A

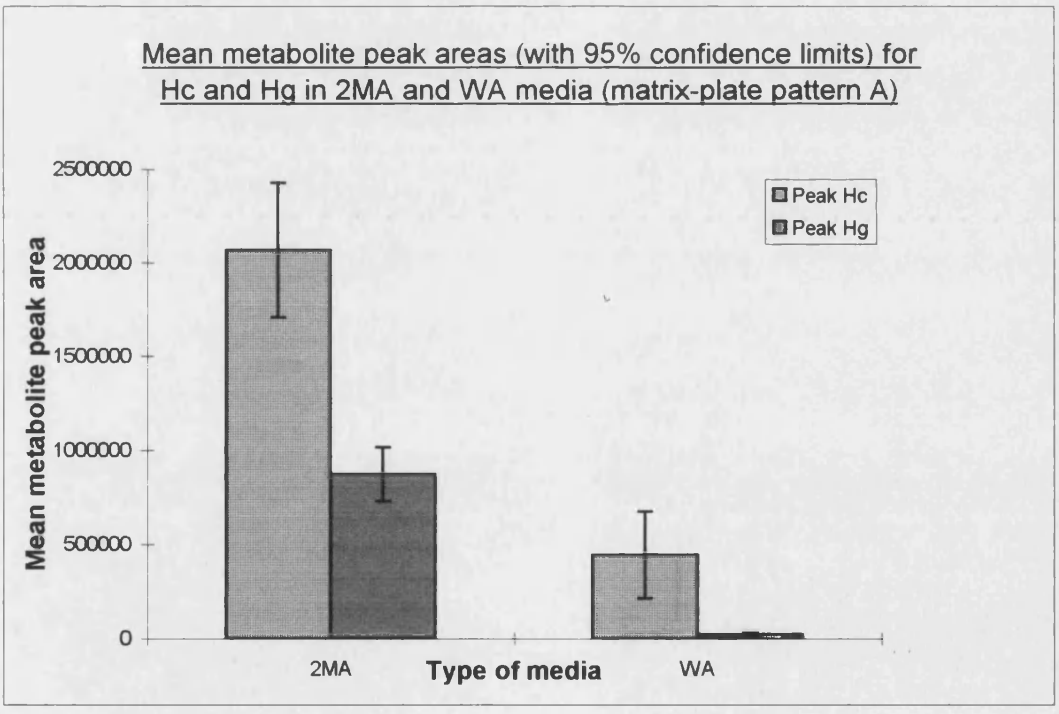


Peak Hg: Matrix H

Key to metabolite peak areas (x 10^4)				
	0			151-180
	0-30			181-210
	31-60			211-240
	61-90			241-270
	91-120			271+
	121-150			

Figure 7.14 : Comparison of metabolite peak Hc and Hg production in 2MA and WA compartments of matrix-plates (55 days)





were recovered in such low amounts (relative to overall metabolite production) from the WA squares in the matrix-plate compartments.

There are two obvious answers to this question; either that the Hg metabolite is not there, or that it is present but has been polymerised into a form resistant to the extraction process. The first possibility may be applicable if Hg is primarily associated with the insulation of aerial mycelium. As this is sparsely produced in the WA compartments, the lack of Hg could be accounted for. The second possibility is that Hg is being produced in the WA compartments, but that is crosslinked into the hyphal boundary as an insulant, rendering it unavailable to the extraction procedure. A useful example of this possibility is provided by the pattern of production of the hydrophobin proteins, which may provide indications of the behaviour of hyphal insulative material. As has been previously discussed, hydrophobins are small, hydrophobic proteins which are constitutively produced and exported by aerial and substrate hyphae in the established mycelium of many fungi (Wessels *et al* 1995). In aerial mycelium hydrophobins accumulate on the surface of the hyphae and self-assemble into insulative 'rodlet layers'. In this state the hydrophobins are extremely difficult to extract compared to most proteins, even though there are apparently no covalent interactions holding them in place. It can be seen from this example that insulative material has the paradoxical property of being least available for analysis when it is performing its most important function. This may apply even more strongly to insulative material composed of secondary metabolite-like molecules, which may assemble (via free-radical activity) into covalently cross-linked melanin-like complexes during the insulative process. Experimental proof of this may be difficult to obtain; one possible experimental avenue would be to follow the insulation process by radio-labelling metabolite monomers,

adding them to a growing culture and then assaying for activity in stringently stripped and washed hyphal walls. The residual activity after such a process may be attributable to labelled metabolites that have become polymerised within the cell wall.

Growth of H.fasciculare in heterogeneous media: General conclusions

H.fasciculare dikaryons show a well-developed ability to exploit the heterogeneous environment of the matrix-plate culture systems. The properties that allow the *H.fasciculare* mycelium to achieve this include the differentiation of mycelial cords and the ability to translocate resources from one compartment to another.

The simple 'flow map' model of nutrient translocation in matrix-plate systems made predictions that correlated with mycelial density data obtained from chequerboard plates. This model made the assumptions that the most translocationally active phase of *H.fasciculare* mycelial growth was during the initial colonisation of compartments, that translocation was acropetal only, and that the pattern of biomass distribution established at this point was fixed thereafter. The second assumption has been shown to be false, as basipetal translocation was observed in matrix-plate designs G and H. The stability of the initially established mycelial density pattern may hold for the matrix-plate systems studied here, but despite their inbuilt heterogeneity matrix-plates are still small batch cultures and the *H.fasciculare* mycelia grown in them are correspondingly limited. Although the flow-map model is flawed and rudimentary, it seems to correlate well with mycelial growth in chequerboard matrix-plate designs and it may be possible to elaborate it further using different matrix-plate experiments to develop a more useful theoretical model for mycelial growth in such a system. An image analysis/matrix-plate system is a promising starting point for the quantitative

study of mycelial dynamics. The establishment of a calibration for biomass in relation to light density is a priority if this is to proceed.

The quantity and proportion of Hc and Hg metabolites produced by the Class 1 dikaryon varied in response to the local nutrient supply and may also vary depending on the translocational processes occurring within the mycelium. The 'flat' Class 2 dikaryon tested (which lacks metabolite peak Hg) showed a reduced ability to form well-differentiated cord networks and exhibited irregular growth dynamics compared to the Class 1 dikaryon; this pattern of growth appeared to be intrinsic to the mycelium rather than responsive to the matrix-plate environment.

Chapter Eight

General Discussion

Heterogeneity and instability in H.fasciculare

The question that was used to initiate this study was this: What are the origins of heterogeneity in *H.fasciculare*? To get a grip on possible answers to this question it may be useful to rephrase the question in terms of stability and instability. It might be stated that *heterogeneity implies dynamics*. If differences are seen within or between the components of a biological system, then some process must be generating them. Heterogeneity arises from instability, from one entity developing or breaking down into others; the opening question might therefore be rephrased as 'what are the origins of stability and instability in *H.fasciculare*?'.

At first glance it might seem that stability is a good thing to have in a biological system, associated with normality and health, and that instability is a bad thing, associated with abnormality and disease. However, stability may be an actual handicap to an organism that is presented with an unpredictable environment, and all developmental processes may be seen as a form of finely controlled instability with an potentially adaptive outcome. To understand the heterogeneity-causing instabilities in *H.fasciculare* mycelia it is necessary to understand the genetic, epigenetic and physiological processes that maintain stability, and then examine both the factors that could alter or undermine the functioning of these processes. A model has been proposed in the opening chapter that attempts to explain mycelial stability in terms of the mycelium's ability to manage oxidative stress, and it is from this viewpoint that this endeavour to understand the heterogeneity within *H.fasciculare* was made.

Most of the variation within the *H.fasciculare* strains studied in later chapters arose during the recovery of cultures from *H.fasciculare* fruit-bodies described in Chapter Three. The processes occurring during this regeneration event are therefore likely to represent the origins of the heterogeneity studied in various ways throughout the rest of this work. Three sets of fruit-bodies from different *H.fasciculare* individuals were used as a source of mycelial cultures; three sets of cultures were recovered from each fruit-body sample. The origin of one of these sets was the natural sexual system that creates basidiospores, the origin of the other two sets was the distinctly unnatural process of subculturing fruit-body tissue and recovering mycelial and conidial cultures from it. In the terms used above, the generation of basidiospores represented a controlled physiological and genetic 'instability' in contrast to the uncontrolled instability during the outgrowth of strains from the fruit-body tissue samples. This uncontrolled instability was probably caused by the multiple stresses (likely to include a significant oxidative stress component) caused by the excision and regeneration of fruit-body tissue. The heterogeneous cultures generated from this included two main classes of dikaryon and several classes of conidial homokaryon.

The analysis of recombinant mating-types provided the only method that could be used to determine the occurrence of genetic recombination processes in basidiospore and conidia-derived homokaryon cultures. The mating-types recovered were found to follow the expected meiotic pattern in the IAC and AV basidiospore sets, but not in the IIAC set, which only had two unequally-distributed mating classes of basidiospore. In this case the natural system for generating genetic heterogeneity seems to have failed. When the conidial isolates from the tissue-regeneration plates were analysed, recombinant strains

were found amongst the IIAC and AV sets, but not the IAC set. The instability within the regenerated tissue cultures was therefore shown to have a genetic element. The varied morphology of the tissue-derived conidial cultures could not be confidently correlated with mating-type, indicating further possible epigenetic or genetic rearrangements beyond those of the mating-type genes.

Origins of the Class 2 dikaryotic phenotype

One of the main distinctions studied in this work was the division between *H.fasciculare* Class 1 and Class 2 dikaryons. These two mycelial phenotypes seemed to share the dikaryotic organisation, but Class 2 dikaryons had reduced aerial mycelium and abnormal pigmentation patterns when compared to the Class 1 dikaryons, which seemed to have a normal, functional phenotype. In Chapter Six the combative abilities of Class 2 dikaryons were found to be reduced compared to Class 1 dikaryons, and a similar reduction in a Class 2 dikaryon's cord-forming abilities was shown in Chapter Seven, along with irregular growth dynamics.

A possible underlying cause for the Class 1/Class 2 dikaryon difference was identified by the HPLC analysis in Chapter Five; this revealed that Class 2 dikaryons had reduced or absent He and Hg metabolite production. These compounds were found associated with the mycelium of Class 1 dikaryons in Chapter Six and have been hypothesised to have an insulative role within the hyphal boundary. If this hypothesis is correct then the oxidative-stress model would predict that a mycelium unable to produce

He and Hg would be subject to a constant, elevated level of oxygen-induced stress; this could certainly explain some of the characteristics of the Class 2 dikaryon phenotype, although whether it is the fundamental cause of the phenotype remains an open question. Since there were no available genetic markers for recombination in the Class 1 and Class 2 tissue-derived dikaryon cultures it was not possible to determine whether genetic recombination processes were responsible in whole or part for the variations between these strains. However, the distinction between Class 1 and Class 2 dikaryons was so commonly expressed and wide-ranging that small-scale genetic rearrangements do not seem to be sufficient to explain it unless they repeatedly occur in a critical area, such as the mating-type genes. The origin of the Class 1/ Class 2 dikaryon heterogeneity may therefore lie at a coarser structural level; possible causes include mitochondrial dysfunction or large-scale genomic disruption or damage. If He and Hg are secondary metabolites, then a disrupted primary metabolism caused by mitochondrial dysfunction could explain why they are reduced in Class 2 dikaryons. If this is the case, then it might be speculated that the irregular growth pattern of the Class 2 dikaryons represents internal changes in the population of functional and dysfunctional mitochondria.

Functions of the Hg and He metabolites

The presence of hydrophobic metabolites has been shown to correlate with a number of the heterogeneities seen within and between the strains of *H.fasciculare* analysed here. As discussed above, the He and Hg metabolites were reduced or absent in Class 2 dikaryons

compared to Class 1 dikaryons. Homokaryons also showed reduced or absent He and Hg peaks when compared to the Class 1 dikaryon profiles. Hydrophobic metabolites were also correlated with differentiation state in the fruit-bodies analysed; the main hydrophilic peak cluster in the fruit-body HPLC traces was relatively more abundant in the cap sections and the main hydrophobic peak cluster was more abundant in the stipe tissue.

Although the difference between the Class 1 and Class 2 dikaryons may be a result of unstable reactions to laboratory manipulations, the difference between homokaryons and dikaryons and the differences within fruit-body tissue are clearly part of controlled developmental processes that may have an adaptive function. If the oxidative stress hypothesis for mycelial differentiation is true, then the distribution of insulative hydrophobic metabolites may have a leading role in these processes. Evidence for He and Hg fulfilling the role of insulants was provided in Chapter Six, where it was found that these metabolites were associated with the mycelium rather than being exported into the growth medium.

Insulation and life-cycle stage in *H.fasciculare*

If the functions of the He and Hg metabolites are those of insulation, then why should the *H.fasciculare* dikaryon, but not the homokaryon, require them? The answer to this may lie in the different internal and external challenges faced by each life-cycle stage. The homokaryon is usually derived from a single spore; it has few resources to conserve and everything to gain if it encounters a nutrient source. This implies an r-selected strategy

where all the mycelial resources are directed at finding and exploiting an unpredictable nutrient supply and assimilating it as fast as possible. In this context it makes little sense to divert resources into conservative, resistive structures. The aerial mycelium of *H.fasciculare* homokaryons is a mass of conidia; this has no apparent adaptive value for invading other mycelial territories or defending the homokaryon resource base; it does seem to have a significant function in the quest to colonise as many primary resource bases and mate with another homokaryon as quickly as possible.

Dikaryons are, by definition, secondary mycelia. This means that they are, at a minimum, already linked to an established, mated homokaryotic mycelium that they can draw upon for resources. They are also, by definition, not the first organism on the scene, so resources are likely to be colonised by competitors that will need to be displaced if the dikaryon is to survive. In addition to these external factors, the dikaryon contains two different types of nuclei that may be experiencing a degree of genomic conflict, and are therefore susceptible to oxidative stress. If external and internal factors are both taken into account it can be seen that the production of insulative compounds (such as He and Hg) for the purposes of conservation, combat and stability would be a useful developmental step. In some ways the homokaryon and dikaryon life stages might be seen to be interdependent. The dikaryon relies on the homokaryon to provide an initial resource base that can then be invested in explorative and combative strategies; the homokaryon relies on becoming a dikaryon to protect and extend its initial, precarious toehold on life.

Heterogeneity between dikaryon genetic individuals of H.fasciculare

The three genetic sources of *H.fasciculare* used in this work represented three individuals of *H.fasciculare*, each successful enough in the natural environment to produce a large crop of fruit-bodies. As three genotypes is a very small sample of the wild population of *H.fasciculare*, it is not possible to draw any conclusions about the degree or origins of variation within the population as a whole. However, the analysis of three different individuals has allowed for a basic understanding of the potentials for this variation and the forms it might take.

The greatest difference evident between the three genetic individuals was that seen between the IIAC individual and the IAC and AV individuals. The IIAC sample may reflect a *H.fasciculare* individual in the early stages of strain degeneration. Although the Class 1 dikaryotic strains recovered from this sample showed strong growth and relatively normal morphology, the homokaryotic cultures isolated from conidia and basidiospores were abnormal when compared to the same cultures from the IAC and AVS samples. The poor, senescent growth of the IIAC basidiospore isolates and their segregation into only two unevenly divided mating type classes (compared to the normal four found in the IAC and AV sets) suggests that the meiotic processes in the fruit-body may be dysfunctional. Conidial cultures recovered from the IIAC tissue outgrowth plates also showed senescent growth and unusual mating-type patterns. Two classes of conidial cultures showed either no mating activity or non-specific mating with both basidiospore mating-type classes. Although mating-type recombination was also found to occur in the AVS individual, it is interesting to note that similar 'universal' mating types have been observed in strains of

Stereum hirsutum harbouring linear plasmid-like sequences in their mitochondrial genomes (Ramsdale and Watkins unpublished data) and that the well-studied senescence processes in *Podospora anserina* have also been linked to such mitochondrial elements (Marbach and Stahl, 1994). These elements have been likened to intracellular parasites; in this context it is interesting that strains of *Cryphonectria parasitica* infected with a 'hypovirulence' virus have reduced pathogenicity, sporulation and pigmentation. Analysis of the gene expression pattern in virus-infected strains of this fungus reveal that only four genes seem to be significantly downregulated (Kazmierczak *et al*, 1996). Two of these genes code for a fungal sex pheromone, one for a laccase and one for a hydrophobin. All of these genes would be predicted by the oxidative-stress model to contribute to boundary-sealing processes; sexual processes involve the production of sealed-boundary reproductive tissues, hydrophobins and laccase are direct architects many other sealed-boundary fungal structures. The benefits to a virus of undermining boundary-sealing processes are obvious; enhanced escape and distribution from its host...the fungal equivalent of a sneeze?

It may be that if such parasitic genetic elements were latent within the IIAC genome then the stress of tissue-regeneration might have activated or selected them, providing another potential aspect to this unstable process.

The lifetime of a particular *H.fasciculare* dikaryon individual in the environment may be protracted due to its indeterminate lifestyle. As usual, the term 'individual' must be used carefully with indeterminate organisms; in this usage it is intended to refer to the dikaryon formed from a particular homokaryon mating, whether integrated or fragmented.

Even without the intervention of genomic or virus-like parasites, genetic damage to both mitochondrial and nuclear genomes may accrue over a period of years and eventually lead to the senescence and eventual death of a particular dikaryotic individual. This may be similar to the processes of strain instability and degeneration by which continuously cultured fungal strains change morphology and metabolite production (Kale *et al* 1994), usually associated with a loss of vigour, and it may also reflect processes of mitochondrial genome damage. Strain instability has been particularly studied in *Agaricus bisporus* where it represents a threat to a commercially important fungal crop (Li et al 1994). There is no reason to suppose that strain degeneration will not occur in 'wild' strains of fungi that are effectively 'continuously cultured' in the environment. Any large sample of natural strains of such fungi will contain a cross-section of the population, including young, old and senescent individuals.

The analysis of any one of the three *H.fasciculare* genetic sources used in this work would have lead to broadly similar conclusions about the distribution and apparent function of metabolites, but would have missed important details. On the other hand, the analysis applied to each of the genetic recombination tests in Chapter 4 would have lead to different conclusions for each genetic source. This underlines the fact that heterogeneity within fungal populations should be taken into account when attempts are made to analyse the basic functions of the mycelium.

This study has shown that the careful analysis of a heterogeneous event (the subculture and regeneration of tissue from a fruit-body) can lead to insights into the organisation and dynamics of an organism. Much of this information was derived from an initially unpromising situation; the transient, confused and chaotic outgrowth from tissue samples. It would have been relatively easy to dismiss this as irrelevant experimental 'noise', subculture the strains to stable homogeneity and then proceed with other experiments. Instead, the heterogeneity was investigated with interesting, and hopefully significant, results.

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